

DESIGN, SYNTHESIS, PHARMACOKINETIC, AND PHARMACODYNAMIC
EVALUATION OF A NEW CLASS OF SOFT ANTICHOLINERGICS

By

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To my parents

Their love makes the impossible possible!

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KEY TO SYMBOLS AND ABBREVIATIONS

AUC	area under the curve
°C	degree centigrade
CDCl ₃	deuterated chloroform
cm	centimeter
g	gram
hr	hour
HPLC	high performance liquid chromatography
i.v.	intravenous
m _{1,4}	cloned human muscarinic subtype receptor (1-4).
M _{1,3}	muscarinic subtype receptor 1-3 from animal tissue (1-3)
mg	milligram
min	minutes
mmol	millimoles
mp	melting point
NMR	nuclear magnetic resonance

pA_2	an empirical parameter defining the negative logarithm of the molar concentration of the antagonist which produces a two-fold shift to the right of a concentration-response curve
ppm	parts per million
QSAR	quantitative structure activity relationship
r^2	regression coefficient
SEM	standard error of mean
$t_{1/2}$	half life
TLC	thin layer chromatography
vs	versus
w/v	weight/volume
μg	microgram
μl	microliter

Abstract of Dissertation Presented to the Graduate School of the University of Florida in
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DESIGN, SYNTHESIS, PHARMACOKINETIC, AND PHARMACODYNAMIC
EVALUATION OF A NEW CLASS OF SOFT ANTICHOLINERGICS

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Chairman: Nicholas Bodor
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The present research involves design, synthesis, and evaluation of a new class of soft anticholinergics with muscarinic subtype receptor selectivity. N-Alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acid, potent anticholinergics with muscarinic subtype receptor selectivity, were chosen as lead compounds. Soft drug design approach based on inactive metabolite were applied to the design.

A receptor binding method was developed for the evaluation of the potency and subtype selectivity of soft anticholinergics using cloned human muscarinic receptors. The soft anticholinergics previously made in our laboratory were evaluated by this method.

Eight compounds were synthesized. The *in vitro* receptor binding studies demonstrated that this new class of soft anticholinergics were able to attain the potency of the lead compound and two of compounds showed muscarinic receptor subtype selectivity. *In vivo* activity of the newly synthesized soft anticholinergics was evaluated by mydriatic studies in rabbits. Two of the compounds were found to have shorter duration of action than that of tropicamide after unilateral administration of equieffective doses. The untreated eyes were found to dilate in tropicamide and other classical anticholinergics treated animals, but not in soft drug treated animals, which indicated a lack of systemic activity of the topically administered soft drugs. The cardiac activities of the soft anticholinergics were evaluated by the ability of protecting the carbachol induced bradycardia effects. Three soft drugs evaluated possessed much shorter protective effects (about 15 to 30 minutes) than that of atropine (at least 2 hours).

In vitro biotransformation studies and *in vivo* pharmacokinetic studies of the soft anticholinergics demonstrated that the newly synthesized soft anticholinergics were hydrolyzed to inactive metabolites and the metabolites were rapidly eliminated from the systemic circulation.

In summary, the soft nature of the compounds, in conjunction with the subtype receptor selectivity, increased the therapeutic index of this new class of soft anticholinergics.

CHAPTER 1 INTRODUCTION

Drug Design

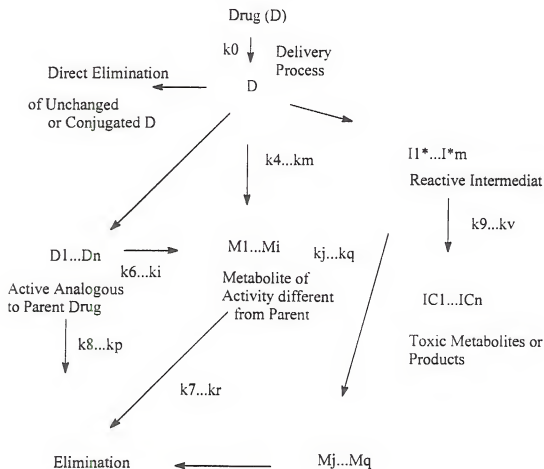
Drugs were discovered by one of the following paths: serendipity, random screening, extraction of active principals from natural sources, molecular modification of known drugs, rational drug design (Korolkovas, 1988), and combinatorial chemistry.

Retrometabolism Approaches of Drug Design

Despite attempts to introduce rational and logical processes in drug discovery, the success ratio is alarmingly low. Very few compounds with maximal or optimal activity will become clinically useful drugs; the main and most frequent reason is the toxicity of these compounds. In conventional drug design, the sole goal of the drug design is to maximize pharmacological activity of the existing compounds. Unfortunately, in most cases, the side effects are related to the intrinsic receptor affinity responsible for the desired activity. It is not surprised that with the increasing of the activity during the drug discovery process, the toxicity of the compounds increase accordingly. The therapeutic index, defined as TD_{50}/ED_{50} , is unchanged. Thus, the main objective of drug design

should not be the activity of the drug but its therapeutic index, the ratio of pharmacological activity to undesired side effects (ED_{50}/TD_{50}). How toxicity are generated during the disposition process?

Toxicity results from a combination of many processes and factors, including not only the other pharmacological effects of the drug itself, but also the various effects of its



Scheme 1. The metabolic fate of a conventional drug after administration.

metabolites, reactive intermediates, and various compounds resulting from direct interactions with cell components. Bodor (1984) has summarized in a scheme (Scheme 1). The toxic effects of a drug as resulting from a combination of factors which include all the other pharmacological effects of the drug (D) itself, the effects of the direct and indirect metabolites ($D_1 \dots D_n$, $M_1 \dots M_j$ and $M_j \dots M_q$, respectively), reactive intermediates ($I^*_1 \dots I^*_m$), and the various compounds ($iC_1 \dots iC_n$) resulting from the interactions of these intermediates with cellular components. The overall toxicity of a drug could be described as the summation of toxicity due to the drug itself, which essentially is its lack of selectivity, and the toxicity due to its various metabolic products.

Since the toxicity is generated from the metabolism of the compounds in the body, it is necessary to include metabolic consideration into the drug design process.

The incorporation of metabolic consideration, or, structure metabolism relationship into the drug design process is the fundamental principle of the retrometabolic drug design.

Retrometabolic drug design approaches include two major methods to improve the therapeutic index of a drug. One is the general concept of chemical delivery systems (CDS). A CDS is defined as a biologically inert molecule that requires several steps in its metabolic conversion to the active drug and that enhances drug delivery to a particular organ or site. This requires multiple enzymatic and/or chemical transformation.

Chemical delivery systems can be divided into several classes as follows:

- (1) enzymatic physical-chemical-based CDS exploit site-specific traffic properties by sequential metabolic conversions resulting in changed transport properties; and (2) site-

specific enzyme-activated CDS exploit specific enzymes found primarily, exclusively, or at higher activity at the site of action.

Soft Drugs

Another major method of retrometabolic drug design is the soft drug approach. The basic principle of soft drug design is to control and direct metabolism by drug design rather than avoid it. Soft drugs are defined as biologically active, therapeutically useful chemical compounds (drugs) characterized by a predictable and controllable *in vivo* destruction (metabolism) to nontoxic moieties after achieving their therapeutic role.

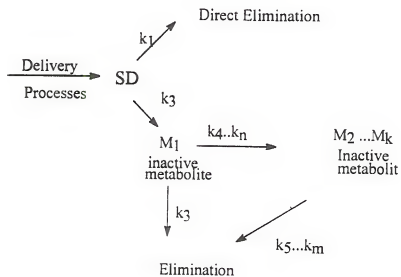
From the earlier discussions of the metabolic fate of drug design after they enter the body, it is obvious that the main purpose of the design of soft drugs is to avoid oxidative metabolism as much as possible. To avoid oxidative metabolism, the soft drug concept advocates the use of hydrolytic enzymes to achieve predictable, controllable, and direct drug metabolism. As shown in Scheme 2, the soft drug (SD) that replaces D simply eliminates the majority of the unwanted process and deliberately simplifies the disposition of the drug. Ideally, the soft drug is inactivated in one step. It is possible for the inactive M1 metabolites to be further metabolized or conjugated again to other inactive products, all of which are eliminated. Thus, the formation of active metabolites and reactive intermediates is avoided.

Approaches to Soft Drug Design

There are several potential strategies to design soft drugs. Based on the current knowledge, there are five distinct approaches for designing soft drugs.

1) Soft analogs

A soft analog is a close structure analog of a known drug, in which a specific metabolically sensitive spot is incorporated, which makes the modified drug undergo a one step detoxification.



Scheme 2. The soft drug concept (Bodor, 1984).

2) Activated soft compounds

In this method of soft drug design, an inactive, nontoxic compound is activated to certain pharmacological function by introducing a pharmacophore group into its structure. The activated soft compound will release the pharmacophoric moiety *in situ* and reverts back to the original nontoxic compound. Thus it serves as a carrier for the pharmacophore.

3) Active metabolites

An active metabolite, preferably in the highest oxidized state, of a known drug is considered to be a soft drug, since it avoids the metabolic pathway which are highly variable among individuals and are subject to modulation by enzyme inducers and inhibitors.

4) Endogenous compounds as natural soft drugs

The human body has efficient metabolic pathways for deactivation of endogenous compounds such as steroids and neurotransmitters. Hence these compounds can be considered as natural soft drugs.

5) Inactive metabolites

In this approach, an inactive metabolite of a known drug is reactivated by structural modification (isosteric and/ or isoelectronic) to resemble the parent compound. The new compound is designed in such a way that *in vivo* it is metabolized in a predictable one-step degradation to the original inactive metabolite.

The Autonomic Nervous System

The nervous system in the human body consists of two major parts: (1) the central nervous system, and (2) the peripheral nervous system. The peripheral nervous system includes the somatic nervous system, or voluntary system, which we are able to consciously control and the autonomic nervous system (ANS), which we are not able to consciously control. The autonomic nervous system coordinates activities of organs that function at the subconscious level, such as respiration, circulation, digestion, metabolism and endocrine gland secretion. The autonomic nervous system is divided into sympathetic and parasympathetic division, based on where the preganglionic nerve originates: sympathetic from thoracic and lumbar region; parasympathetic from cervical and sacral regions. The sympathetic fibers ramify to a much greater extent than the parasympathetic fibers. This diffuse discharge of the sympathetic nervous system can prepare an organism for the "fight or flight" reaction. The parasympathetic nervous system, however, generally displays a 1:1 ratio between preganglionic and postganglionic fibers. It is prepared for the discrete, local discharge of neurotransmitters and is primarily involved in the conservation and restoration of energy needed for everyday bodily functions. Any blocking of its transmission will have the opposite effect as activation of the ANS. In the most common situation, sympathetic division enhances the activity (e.g., heart rate), whereas the parasympathetic system decreases the activity.

Anticholinergic Agents

Anticholinergic agents competitively inhibit the actions of acetylcholine by blocking the interaction of this endogenous neurotransmitter with its receptor. This process can occur at autonomic effectors innervated by postganglionic nerves, as well as on smooth muscles that lack cholinergic innervation. Atropine is the prototype for drugs which antagonize the muscarinic activities of acetylcholine. Atropine and its analogs have very little effect at nicotinic acetylcholine receptor sites and thus this type of compound is referred specifically as antimuscarinic.

The structural elements of cholinergic antagonists (Wess, 1990) are (1) a cationic "head group" which is either a tertiary base which is protonated at physiological pH or a quaternary ammonium moiety; (2) some "heavy blocking moieties," e.g., alicyclic or aromatic rings, for hydrophobic interaction with the receptor; (3) an interconnecting structural element (ester or amide) of definite length; and (4) an "anchoring group," e.g., hydroxyl group(s) are often present at key positions.

Figure 1-1 showed two typical structures of classical anticholinergics, where the ester oxygen and the quaternary head are separated by 2 or 3 carbon atoms, respectively. It is generally believed that muscarinic receptors required at least two carbon atoms separating the quaternary head and the ester oxygen in order to have significant binding and activity.

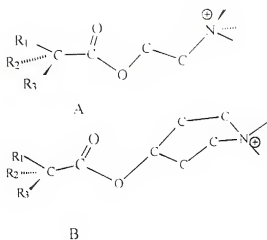


Figure 1-1. Two typical structures of classical anticholinergics.

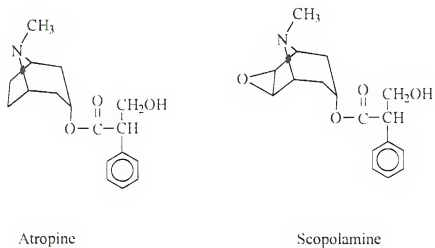


Figure 1-2. Structure of atropine and scopolamine.

Figure 1-2 shows the structures of atropine and scopolamine, the two most frequently used anticholinergics. They belong to belladonna drugs. Atropine and scopolamine are organic esters formed by combination of an aromatic acid, tropic acid, and a complex organic base, either tropine or scopine. Scopine differs from tropine only in having an epoxyl group in tropine structure (Figure 1-2). The intact ester of tropine and tropic acid is essential for the antimuscarinic action of atropine, since neither the free acid nor the base exhibit significant anticholinergic activity. The presence of a free OH group in the acid portion of the ester also is important for activity. Substitution of other aromatic acids for tropic acid modifies but does not necessarily abolish the anticholinergic activity.

Pharmacology of Anticholinergic Agents

In the eye, the pupillary constrictor muscle is dependent on muscarinic cholinergic activation. This activation is effectively blocked by topical atropine and other tertiary antimuscarinic drugs and results in mydriasis. The second important ocular effect of antimuscarinic drugs is weakening of contraction of the ciliary muscle, or cycloplegia. In the Cardiovascular system, in the presence of low dose atropine (antimuscarinic agents) causes parasympathetic stimulation and results in bradycardia. Moderate to high therapeutic doses of atropine cause tachycardia. In the respiratory system, both smooth muscle and secretory glands of the airway receive vagal innervation and contain muscarinic receptors. The antimuscarinic agents cause bronchodilation and reduction of secretion. In the gastrointestinal tract, antimuscarinics reduce the volume of saliva and produce dry mouth. They also reduce both the volume and the total acid

content of gastric secretions. Atropine-like drugs inhibit the activity of sweat glands, causing anhydrosis.

Therapeutic Use of Anticholinergics

Antimuscarinic compounds have been used primarily in the treatment of peptic ulcer disease and irritable bowel syndrome. However, they also have been utilized effectively as bronchodilators, antiperspirants, preoperative medications to inhibit secretions of the respiratory tract, and prophylactics in the prevention of motion sickness. Antimuscarinics have also been employed in the management of Parkinson's disease and as ophthalmic aids.

Development of Anticholinergic Agents

The most common toxicities associated with the use of anticholinergic compounds are due to the indiscriminate binding of these drugs to all muscarinic receptors, thereby eliciting both wanted and unwanted pharmacological effects. Muscarinic receptors have four subtypes. Each subtype receptor has specific action and particular locations in the human body (For review see a later part of this chapter). Cholinergic antagonists will reduce the tone and motility of the stomach as well as decrease gastric secretion, and also produce dry mouth, blurred vision and tachycardia (Brown and Taylor, 1996). The frequency and severity of adverse effects are generally dose-related. Therefore, a reduction in dosage may attenuate unwanted side effects, but may also reduce any potential therapeutic effect. Infants, geriatrics, and patients with Down's syndrome are particularly sensitive to the actions of this class of drugs. In

addition, patients with open-angle glaucoma may be especially susceptible to increased intraocular pressure when exposed to these compounds (Greenstein et al., 1984).

In view of the toxicities related to the anticholinergics, more potent and safer anticholinergics are needed. To address the safety issue, the obvious and direct approach would be searching anticholinergic agents that specifically bind to the particular receptor which elucidate the desired effects. This is a pharmacodynamic approach. The strategy to achieve this goal is to study the molecular structure of the muscarinic subtype receptor, in conjunction with studying of quantitative structure activity relationship (QSAR) of the existing anticholinergic agents in order to design new class of anticholinergic agents with muscarinic subtype selectivity. Another approach is to confine the desired anticholinergic action in the ideal location. This is a pharmacokinetic approach. The strategy to achieve this goal is through retrometabolic drug design (soft drug design).

Searching for Subtype Selective Anticholinergic Agents

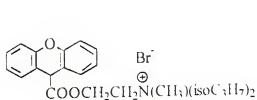
A search for compounds that only bind to the receptors which are responsible for the desired effects was initiated decades ago (Korolkovas, 1988). In an effort to reach this goal, numerous compounds have been synthesized based on atropine as a lead. These are the first group of synthetic anticholinergic agents. This pioneer work has led to realization of the quaternization of the nitrogen atom of atropine changing the pharmacokinetic characteristics of the drug. This alternation allowed for the reduction or elimination of the CNS effects. However, the new quaternary ammonium compounds displayed nicotinic as well as muscarinic receptor blocking activities (Kirsner et al., 1957). In order to further increase pharmacological selectivity and decrease toxicity, many

amino alcohol esters of substituted acetic acids, not based on atropine, were synthesized. The compounds were later tested for biological activity, particularly for the clinically useful antisecretory properties.

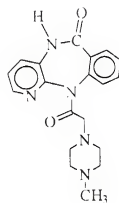
The gastric antisecretory effects of 35 of these quaternary ammonium compounds were tested for potency and for occurrence and severity of side reactions. Parallel trends in activity and toxicity were apparent throughout the study. The drugs which displayed good antisecretory efficacy also produced moderate to severe side effects. In this category were compounds such as methylscopolamine, propantheline bromide and hexocyclium methylsulfate. Unfortunately, the use of antisecretory compounds which produced adequate symptomatic relief often had to be discontinued due to the development of undesirable side action.

In recent years, some progress has been made in the development of anticholinergics with subtype selectivity. A few compounds have reached clinical trials; some of them have been approved for clinical treatment for related disease (Eglen and Watson, 1996).

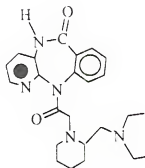
Pirenzepine, an antagonist with relatively high affinity for the muscarinic M_1 and modest affinity for the muscarinic M_4 receptor, is approved for clinical use in the treatment of peptic ulcer disease (Carmine and Brogden, 1985; Hirschowitz et al., 1995). Structurally related compounds in clinical development include telenzepine and nuzenvzepine (Eglen and Watson, 1996). BIBN 99, a lipophilic, centrally acting



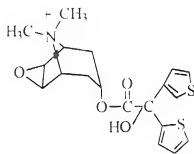
Propantheline



Pirenzepin



AF-DX 116



Tiotropium

Figure 1-3. Structures of some anticholinergics currently in the market.

muscarinic M_2 receptor antagonist, may be useful in the treatment of Alzheimer's disease, since it could reverse the autoinhibitory control of acetylcholine release (Doods et al., 1993). Conversely, peripherally acting muscarinic M_2 receptor antagonists, such as AF-DX 116 (Otenzepad) may be useful in the treatment of bradycardia (Schulte et al., 1991). Selective blockade of muscarinic M_1 receptors may be therapeutically useful in the treatment of respiratory disorders, such as chronic obstructive airway disease, gastrointestinal disorders, such as irritable bowel syndrome (Wallis, 1995), and urinary tract disorders, such as urge incontinence (Taira, 1972; Andersson, 1993). In terms of obstructive airway disease, stimulation of cholinergic nerves provides the major bronchoconstriction control of animal and human airways (Morley, 1994). Tiotropium is a novel antagonist with a preferential slow off from muscarinic M_1 receptor with respect to muscarinic M_2 receptors (Maesen et al. 1993; Haddad et al. 1994). Newer compounds, with selectivity for the M_1 and M_3 receptors over M_2 or M_4 receptors include zamifenacin and the structurally related, darifenacin (Houghton et al., 1993; Wallis, 1995; Wallis et al., 1995). Darifenacin is a selective muscarinic receptor antagonist in phase II clinical trial for both urinary incontinence and irritable bowel syndrome (Wallis et al., 1995). Vamicamide is also a novel compound under development for the treatment of urinary incontinence (Oyasu et al., 1994). It is selective toward muscarinic M_1 and M_3 receptors over M_2 receptor *in vitro*. *In vivo*, vamicamide dose-dependently inhibits spontaneous bladder contractions caused by elevation in the intravesical volume. At a 3-10 fold higher dose, no effect was seen on the contractions of stomach or colon, response also mediated via activation of muscarinic receptors. In summary, several advances have been made in the identification of antagonist that can discriminate between muscarinic receptor

subtypes. Most research have centered upon selective muscarinic M_1 antagonist, by virtue of their therapeutic potential in the treatment of smooth muscle pathophysiology. To date, most of these compounds discriminate well between M_1 and M_2 receptors, and less so between M_1 and M_3 receptors.

Soft Anticholinergics

Besides through searching for anticholinergics with subtype selectivity to improve therapeutic index of the anticholinergics, soft drug design concept has been successfully applied to design safer anticholinergics (Kumar and Bodor, 1996).

Of the five basic methods for the design of soft drugs (see Soft Drug section), soft analogue and inactive metabolite approaches were the two adopted to design soft anticholinergics. Bodor et al. (1980) reported design and synthesis of a series of "soft" anticholinergics based on soft analog approach. It was generally believed that the muscarinic receptors required at the least two carbon atoms separating the quaternary head and the ester oxygen in order to have significant binding (Figure 1-1). However, Bodor et al. shortened two carbon bridge of the conventional anticholinergic agents to one carbon to incorporate a metabolic sensitive spot into the structure of "soft anticholinergics". This new series of soft analogs of conventional anticholinergics were proved to be the potent anticholinergic agents which were hydrolyzed *in vivo* in a predictable time frame to an acid, an aldehyde, and a tertiary amine acid (Figure 1-4).

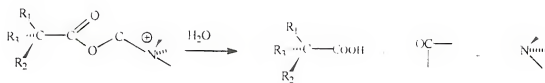


Figure 1-4. Hydrolysis of soft analogs of conventional anticholinergics.

The soft analog concept was also applied to the design of soft propantheline (Brouillette et al., 1996). The ethylene bridge of propantheline (Figure 1-3) was shortened by one carbon to produce a new series of compounds retaining of anticholinergic activity and decreased hydrolytic stability.

Inactive metabolite approach has been applied to the design of several series of soft anticholinergics. Hammer et al. (1988) used inactive metabolite soft drug design principle to design soft anticholinergics based on atropine. Hammer chose a hypothetical metabolite of atropine, an oxidation product of the primary hydroxyl group, as the lead compound. This lead compound was reactivated by esterification with aliphatic and cycloaliphatic alcohol of varying chain length. The resultant compounds exhibited similar activity as atropine but with less duration in the biological media. One of the resultant compounds, tematropium methyl sulfate, has actually been able to reach phase II human trial as short-acting mydriatic diagnostic agent. Tematropium is also currently undergoing human development as a safe antiperspirant.

The inactive metabolite principle for the design of soft drugs was also applied to design soft drugs based on methscopolamine (Kumar et al., 1993a). Hypothetical carboxylic metabolite of methscopolamine and scopolamine were chosen as the lead

compounds. The lead compound was reactivated by esterification with various alcohols to produce a series of soft drugs. Soft drugs of methylscopolamine were found to be potent anticholinergics in both *in vitro* and *in vivo*. All soft drugs examined were metabolically more unstable than methscopolamine in all the biological media tested, indicating that soft drugs will be cleared from systemic circulation at faster rates than methscopolamine, thereby minimizing the systemic side effects.

As a general principle, Hammer et al. and Kumar et al.'s findings indicated that the introduction of the metabolically sensitive ester function in the atropine or scopolamine analogs, resulted in soft anticholinergics with somewhat reduced intrinsic activity. For example, tematropium showed a pA_2 of about 0.6 to 0.8 log unit lower than that of its parent compound. Based on the observation that in the soft analog class, such as $(\pm)-[(\alpha\text{-cyclopentylphenylacetoxymethyl})\text{triethylammonium chloride}]$ the introduction of a cyclopentyl group in the acidic component did enhance activity (Bodor et al., 1980), our laboratory tried to introduce a cyclopentyl group into the tematropium molecule (Juhasz et al., 1998). However, the resulting ethoxycarbonylphenylcyclopentylacetyl-*N,N*-dimethyltropinium methyl sulfate (PCMS-1) and methoxycarbonylphenylcyclopentylacetyl-*N,N*-dimethyltropinium methyl sulfate (PCMS-2) were shown somewhat less potent than tematropium. A possible reason for the resulting compounds being less potent than expected is that the introduction of the bulkier group, e.g., cyclopentyl group, enhanced the activity of the anticholinergics, but such enhancement was compromised by the molecular volume increase: The molecular

volume is one of the major factors contributing to the activity of soft anticholinergics (Kumar et al., 1994).

Radioligand Binding As a Fool in Drug Discovery

The ultimate goal of drug discovery is to develop therapeutic agents which prevent, alleviate, or cure human disease states. In order to meet this goal, an efficient and reliable pharmacological method must be established to test the newly synthesized compounds. Historically, animal models, or systemic screening tests were heavily employed as the valid tools to predict the potential therapeutic utility. But, the systemic test has two major limitations: (1) It is not efficient. Generally speaking, it takes days or weeks to accomplish a test which is becoming a rate-limiting point in the drug discovery process. (2) It does not provide information of molecular mechanism of drug action.

Conceptually, it is not difficult to understand why animal model basis of pharmacological tests is difficult to work with. It is the nature of animal models that they are complex and the dynamic arrangement of molecular mechanisms limits their ability to selectively target one mechanism of action.

Mass screening tests, most of which are based on radioligand binding, have been proved to be fast and dependable methods for screening. They also provide very valuable information on the mechanism of action of the particular drug during the process of evaluation of pharmacological activity. Thus it has produced a shifting of priorities and attitudes from "systems" screening towards mass ligand screening strategies, which offer

a rapid, efficient, and reliable means of identifying compounds on the basis of mechanism of action information (Sweetam et al., 1995).

Principles of Drug-Receptor Interactions

Our current concepts of receptors have their origins in the work of Paul Ehrlich (1845-1912) and J.N. Langley (1852-1926). Both Ehrlich and Langley drew attention to a most important feature of drug action chemical specificity, or mutual recognition of drug and receptors.

Not all sites with which drugs are able to bind are necessarily receptors. The current concept of a receptor is of a macromolecule with which a drug interacts, leading to a change in cellular function. Thus the concept of a receptor pharmacologically includes both the capacity to bind to or react with a drug and to mediate both the positive and negative biologic alteration in function. The connection of receptor to the inner cellular function is the integral part of this important concept. The old term acceptor has been used to describe sites to which a drug can combine but not cause a biologic change.

Affinity is used to describe the propensity of a drug to bind at a given receptor site; intrinsic activity describes its ability to initiate biologic activity as a result of a binding. Presumably because of the complexity of the binding process, a drug may possess affinity, that is, be able to bind to a binding site, yet not initiate specific activity. But, in the current receptor binding studies, we assume that the drug with high affinity toward a particular receptor will show a high specific pharmacological activity. Two chemically similar drugs that initiate the same selective activity probably do so by acting

on the same population of receptors. If one is effective at the lower molar concentration than the other it is said to be more potent.

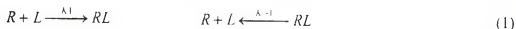
Ligands for a receptor can be specified as agonists and antagonists. An agonist is a drug, hormone or neurotransmitter substance that elicits a cellular response when it combines with a receptor. A full agonist is capable, at sufficient high concentration, of producing a maximal cellular response whereas a partial agonist's maximum effect is less than the maximal response of which the tissue is capable. An antagonist is a drug which prevents the effect of an agonist by combining with the same receptor without causing activation. Antagonist can be reversible or irreversible, and competitive or non-competitive. Ligands not only differ in their ability to produce an effect upon drug-receptor complexation, but full agonists have an intrinsic activity of one and antagonist have an intrinsic activity close to zero, but also differ in their affinity for a receptor.

Kinetics of Drug-Receptor Interactions

Numerous mathematical, thermodynamic, and biochemical models have been put forth to describe the interactions of drugs with their receptors. The preeminent theory from the point of view of attempting to describe drug-receptor interactions has been the occupation theory, in which a response is thought to emanate from a receptor only when it is occupied by an appropriate drug molecule. This model is the first proposed and its historical development traces the essential elements of drug-receptor interactions. Another model termed the rate-theory, equates drug-receptor activation with the kinetic rate of the offset of drugs and describes activation in terms of kinetics rather than binding. A model that bridges these two approaches is called the inactivation model. The two-state model

suggest that the binding of a drug molecules to a receptor is not an independent process in the sense that the binding to the receptor by one drug molecule is believed to affect subsequent binding of another drug (Kenakin, 1993).

When a ligand (L) binds to a single class of noninteracting binding sites (R), the following equilibrium exists:



where K_1 is the rate constant for the association and K_{-1} is that for the dissociation of the receptor-ligand complex. The equilibrium dissociation constant for this reaction is given by,

$$K_d = \frac{[R][L]}{[RL]} \qquad (2)$$

The affinity constant is defined as

$$K_a = \frac{1}{K_d} \qquad (3)$$

$$\text{At the equilibrium, } \frac{d[RL]}{dt} = K_1[R][L] - K_{-1}[RL] = 0 \qquad (4)$$

$$\text{which means } \frac{[R][L]}{[RL]} = \frac{K_{-1}}{K_1} = K_d \qquad (5)$$

The proportion of occupied receptors can be expressed as

$$\frac{[RL]}{[R]_{tot}} = \frac{[RL]}{[RL] + [R]} = \frac{\frac{[R][L]}{K_d}}{\frac{[R][L]}{K_d} + [R]} = \frac{[L] / K_d}{[L] / K_d + 1} = \frac{[L]}{[L] + K_d} \qquad (6)$$

This means that K_d represents the ligand concentration at which 50% of the receptor is occupied.

In order to determine the affinity of a compound for its isolated receptor, one can design an *in vitro* competition type of binding experiment. The receptor preparation is incubated with increasing concentrations of a nonradioactive drug (D) and a fixed low concentration of radioactive ligand (L^*). The advantage of this method is that no radioactivity need to be used in the synthesis of the compound of interest (D). In a system with saturable, reversible binding, bound radioactivity will decrease as nonradioactive drug competes with L^* for a fixed number of binding sites (R_{tot}). For a radioactive ligand concentration, low enough to act as a tracer for unoccupied sites ($[L^*] \ll K_d$), the concentration of nonradioactive drug necessary to replace 50% of L^* from RL^* (IC_{50}) should approximate the K_d for this competing drug (K_i).

In the absence of nonradioactive ligand (D), R and L^* exist in the following equilibrium:

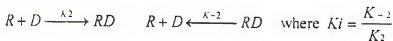


$$\text{where } K_d = \frac{K_1^{-1}}{K_1}$$

and the proportion of occupied receptors equals

$$\frac{[RL^*]}{[R_{tot}]} = \frac{[L^*]}{[L^*] + K_d}$$

In the presence of D, however, one additional equilibrium has to be taken into account:



$$\text{and } R_{tot} = [R] + [RL^*] = [RD] = [R] + \frac{[R][L^*]}{K_d} + \frac{[R][D]}{K_i} \quad (7)$$

So that the proportion of receptors occupied by L^* becomes

$$\frac{[RL^*]}{[R_{tot}]} = \frac{[L^*]}{[L^*] + Kd(1 + D/Ki)} \quad (8)$$

If the assay is set up under such conditions so that the receptor concentration is low compared to that of the ligand, then the ligand concentration $[L^*]$ remains essentially unchanged in the presence or absence of drug and/or receptor ($[L^*]$ in equation (6) and (8) become identical). At a drug concentration of IC_{50} , where the ratio of the proportion of $[RL^*]$ in the presence of D to the proportion of $[RL^*]$ in the absence of D is half, the following equation can be set up:

$$\frac{\frac{[L^*]}{[L^*] + Kd(1 + IC_{50}/Ki)}}{\frac{[L^*]}{[L^*] + Kd}} = \frac{1}{2} \quad (9)$$

$$\text{From this equation, we can have : } Ki = \frac{IC_{50}}{1 + [L^*]/Kd} \quad (10)$$

Thus, if we know the IC_{50} of the second drug (D) at the competition and the dissociation constant and concentration of the radioactive ligand, it is easy for us to derive the Ki (dissociation constant) of the competitive agent (D).

Basic Methodology of Receptor Binding

We have two basic methods for receptor binding studies: (1) saturation receptor binding, which requires the tested ligands to be radio-labeled, and (2) competition receptor binding. The compounds need to be tested are not radio-labeled. They compete with the known radioligand for the same binding sites of the receptors. When reaching the

equilibrium, the percentage replacement of the known radioligand will be used to calculate the K_d (dissociation constant) of the tested ligand.

(1) Saturation experiment ----finding the dissociation constant of the radioligand

By definition, a saturation experiment adds increasing amounts of radioligand to a fixed amount of tissue preparation and measure of the resulting binding. The goal is to measure the dissociation constant.

$$a = \frac{B_{\max} * X''}{K_d + X''}$$

where

a: specifically bound of radioligand, [3H] NMS.

B_{\max} : maximum binding site

X: concentration of radioligand, [3H]NMS

K_d : dissociation constant of radioligand.

(2) Competition experiment

The most common type of binding experiment is the addition of increasing concentration of nonradioactive drug to a fixed low concentration of radioactive ligand and tissue. By measuring the displacement of the radioligand (filtration), we are able to make a curve of displacement.

$$\% A(bound) = 100 - \frac{B''}{IC_{50}'' + B''}$$

$$Ki = \frac{IC_{50}}{1 + \frac{[A]}{Kd}}$$

where

A: radioligand

Kd: dissociation constant of radioligand and receptor complex.

Ki: affinity constant of antagonist and receptor complex.

Muscarinic Receptors

In 1914, Sir Henry Dale discovered two different actions of acetylcholine. One was selectively mimicked by muscarine and blocked by atropine. The other was mimicked by nicotine and blocked by d-tubocurarine. This led to the concept of different subtypes of cholinergic receptors, referred to as muscarinic and nicotinic receptors. Muscarinic receptors mediate most of the inhibitory and excitatory effects of acetylcholine (ACh) on central neurons and the majority effects of ACh in the periphery (see Buckley and Caulfield, 1992 for review). By 1980, it was realized that the muscarinic receptor-mediating action could not be accounted by a single receptor subtype. In particular, radioligand experiment demonstrated that tissue-specific differences in the affinity of the antiulcer drug pirenzepine, suggested the presence of at least two receptor subtypes, M₁ and M₂ (Hammer et al., 1980). However, when taking account for the discrimination observed with 4-diphenyl acetoxy-methyl piperidine methiodide (4-DAMP) between the "M₂" receptors in heart and ileum (Barlow et al.,

1976), it was apparent that at least three muscarinic-receptor subtypes were needed to account for the data. New compounds hexahydro-sila-difenidol (HHSiD) and its para-fluoro analogue (p-F-HHSiD) were developed by Lambrecht, Mutschler, and Tacke (Mutschler and Lambrecht, 1984). The compounds exhibited a 70-fold higher affinity for smooth muscle and glandular muscarinic receptors than for cardiac muscarinic receptors in functional *in vitro* experiments and in radioligand binding studies. Further evidence for the heterogeneity of muscarinic receptors with a low affinity for pirenzepine was obtained from studies on potent at the inhibitory autoreceptors in the guinea-pig ileum (M_3) rather than the inhibitory heteroreceptors in the rat heart (M_2).

In summary, the antagonists selectivity for M_1 , M_2 , M_3 , and M_4 are listed as follows.

M_1 receptors have been defined as those with high affinity for pirenzepine and low affinity for compounds such as AF-DX 116; this is not an adequate definition, as there is significant overlap between affinities of pirenzepine for M_1 and M_3 receptors. Himbacine is currently the only antagonist that can differentiate between M_1 and M_3 receptors. (Lazareno et al., 1990; Caulfield and Brown, 1991; Bernheim et al., 1992).

M_2 receptors are usefully defined by high affinity for methoctramine (7.9-8.3) and low affinity for pirenzepine (6.3-6.7), 4-DAMP (8.2-8.4) and Para-Fluoro-hexahydrosiladifenidol (p-FHHSiD :6-6.9).

M_3 receptors have high affinity for 4-DAMP and p-FHHSiD (7.8-7.9) but low affinity for pirenzepine (6.7 -7.1).

M_4 receptors can now be defined as having moderate (binding experiments : 7.2-7.6) to high affinity (functional experiments: 7.7. Caulfield and Brown, 1991; Bernheim

et al., 1992) for pirenzepine and high affinity for himbacine (8-8.5).

Molecular cloning of muscarinic receptors has established the presence of five receptors as m1, m2, m3, m4, and m5 (Bonner et al., 1987; Peralta et al., 1987; Bonner et al., 1988). Most likely that the functional classification of muscarinic receptor (M_1 - M_5 , maybe M_1) is corresponded well with cloned receptors (m_1 - m_5) (Hulmet et al., 1990; Dorje et al., 1991; Caulfield, 1993).

M_1 receptor predominantly exists in the brain, which is involved in behavioral and cognitive functions (Hammer and Giachetti, 1982; Watson et al., 1983). The heart is one of the rare tissues where only one of the subtype muscarinic receptor presents: M_2 , (Caulfield, 1993; Waelbroeck et al., 1989). In secretory glands the muscarinic receptors mediating enhancement of secretion are the M_3 subtype. M_1 also occurs in the smooth muscles of airways, the gastro-intestinal tract and the urinary bladder (Mutschler and Lambrecht, 1984; Doods et al., 1987). The physiological function of M_1 has not been elucidated yet even though the protein of M_1 receptor has been found in the peripheral lung strip of the rabbit (Dorje et al., 1991; Lazareno et al., 1990).

Pharmacokinetics of Anticholinergics

Although anticholinergic drugs have been used in clinical anesthetic practice for many decades, their detailed pharmacokinetics have been evaluated only during the last few years due mainly to the latest development of new analytical methods for drug determination.

Determination of Anticholinergics in Biological Fluid.

Several methods have been used to measure anticholinergics in humans (Ali-Melkkila et al., 1993). Regarding atropine, a few of radioimmunoassays (RIA) with sufficient sensitivity for clinical pharmacokinetic studies have been published (Wurzburger et al., 1977; Berghem et al., 1980; Virtanen et al., 1980; Ellinwood et al., 1990). The sensitivity of such assay is to be able to reach 1 ng/ml. However, RIA method may have different cross reactivity to D-hyoscyamine and L-hyoscyamine from lot to lot. This could explain why the C_{max} value of atropine with the same dose varies so much from experiment to experiment (Xu et al., 1995).

Radioreceptor assay (RRA) can be applied to the bioanalysis of all anticholinergic drugs normally used in clinical practice. The principle of RRA is based on the competition between drug and a radiolabelled ligand for binding to a certain receptor. When a competitive drug is added to the mixture containing fixed amount of receptors and a radiolabelled ligand, the drug will displace a certain amount of the labelled ligand, depending on the concentration and dissociation constant. The actual concentration of the drug can be calculated from the logit-log transformation standard line. The RRA monitors the drugs reacting with the cholinergic receptor *in vitro* at the muscarinic binding site and therefore, only the biologically active components of an anticholinergic agents will be measured. Thus, the concentration measured by RRA are more likely to correlate with the pharmacological effect compared with chemical methods. On the other hand, RRA has its own limitation. It measures all drugs and their components that are capable to bind to muscarinic receptors. Consequently, it can not discriminate between the parent drug

and the possible active metabolite, and the other drugs with anticholinergic activity can interfere with the assay.

Radioreceptor assay (RRA) has been successfully applied to the studies of pharmacokinetics of atropine (Metcalf, 1981; Aaltonen et al., 1984), scopolamine (Cintron and Chen, 1987), ipratropium (Ensinger et al., 1987), oxyphenonium (Ensinger et al., 1984), and tropicamide (Vuori et al., 1994). Ipratropium and oxyphenonium are quaternary ammonium muscarinic antagonists.

Okuda et al. (1991) developed a high performed liquid chromatography (HPLC) method for the determination of atropine in biological specimens. The samples were extracted by methylene chloride then reconstituted with mobile phase. The retention time of atropine could be varied by either changing the acetonitrile-water ratio in the mobile phase or the pH of the mobile phase. The required sample volume was 2 ml. The detection limit was 8.5 ng/ml. It is the most successful HPLC method development for the determination of atropine in plasma. GC/MS methods has also been published (Hinderling et al., 1985a; Kehe et al., 1992) for the detection of anticholinergics in biological fluid. Hinderling's methods were to extract atropine by chloroform then hydrolyze it into tropine. Subsequently, tropine was converted into its heptafluorobutryl derivative which was measured by GC-MS. The sensitivity was 0.5 ng/ml. The methods were very tedious due to the complicated extraction and derivation methods. This method has been successfully applied to the studies of PK/PD modeling of atropine. Kehe's method has a limit of quantitation of 1.0 ng/ml with 1 ml sample. A simple liquid chromatography/tandem mass spectrometric (LC/MS/MS) method (Xu et al., 1995) was developed and validated to facilitate the pharmacokinetic studies of L-hyoscyamine. This

method utilized a methylene liquid/liquid extraction and gave a limit of quantitation 20 pg/ml with 1.0 ml of human plasma. However, an expensive instrument, tandem MS, was needed.

Pharmacokinetics of Anticholinergics

Most available data of pharmacokinetics of anticholinergics are derived from human studies.

Absorption, elimination, and metabolism

The tertiary amines, atropine and scopolamine, are absorbed relatively well from the gastro-intestinal tract. An oral dose of atropine is rapidly absorbed from the mucosal surfaces and from the intestine, but not from the stomach (Beerman et al., 1971). The gastrointestinal absorption of the quaternary amines, like glycopyrrolate, appears to be slow and erratic (Ali-Melkkila et al., 1991). It is likely due to the fact that it is difficult for the positive charged quaternary amine to cross the biological membrane.

The various chemical analytical methods used have resulted in a highly variable picture of the metabolism and excretion of atropine (Wurzbarger et al. 1977). Atropine and glycopyrrolate are excreted to a great extent as unmetabolized parent agent and/or as pharmacologically active metabolites capable of binding to muscarinic receptors *in vitro* (Ali-Melkkila et al., 1990; Ensing et al., 1984; Kentala et al., 1989).

Fifty-seven percent of atropine (gas chromatographic-mass spectrometric assay) excreted in the urine as unchanged drug, and 29% was excreted as an inactive metabolite, tropine (Hinderling et al., 1985a and Hinderling et al., 1985b). Kalser and McLain

(1970) found that thirty percent of atropine was metabolized (tropine-labelled as [^{14}C]-atropine).

Intravenous administration

Atropine has a short distribution half-life of approximate 1 min and there is a rapid decline in concentration within the first 8-10 min. It has been calculated that the amount of atropine remaining in the circulation at 10 min after iv. injection corresponds less than 5% of the administered dose (Berghem et al., 1980). Atropine is widely distributed in tissue, the apparent volume of distribution ($V\beta$) is over 1 l/kg (Adams et al., 1982; Aaltonen et al., 1984; Hinderling et al., 1985a; Hinderling et al., 1985b; Thiemann et al., 1996). The total clearance is 10 to 15 ml/min/kg. The half life is about 2 hours (Ali-Melkkila et al., 1993). However, the different method for the determination of atropine in plasma resulted in significant difference in the pharmacokinetic parameter (Aaltonen et al., 1984; Kentala et al., 1990; Thiemann et al., 1996). The radioreceptor assay (RRA) only detects the active l-hyoscyamine, on the other hand, the radioimmunoassay (RIA) and other chemical chromatography methods determined both d- and l-hyoscyamine, thus, the plasma concentration determined by RIA is significantly higher than that of RRA. So is AUC. As a result, the clearance and volume distribution calculated from RRA are significantly higher than that of RIA. So, one should be cautious when comparing the pharmacokinetic parameters determined by one method to another method. This is particularly important when new sensitive chemical analysis (HPLC-MS/MS) is replacing the traditional bioassay. Due to the different distribution behavior of d- and l-hyoscyamine, only two distinct phases were seen in the serum curve as

determined by RIA where the levels measured by RRA were well fitted by the 3-compartment open model (Aaltonen et al., 1984).

A few of pharmacokinetic studies of atropine in other species were reported. Urso et al. (1991) reported the studies of pharmacokinetics of atropine after i.v. in rats with RRA. They found that the clearance was 58 ml/min/kg, and the volume of distribution of central compartment was 3 l/kg. Thiermann et al. (1996) studied pharmacokinetics of atropine in dogs with RRA method to determine the concentration of atropine in plasma. The clearance was found to be 44 ml/min/kg, which is much higher than the value of 13.5 ml/min/kg reported by Wurzbarger et al. (1977). It was believed (Thiermann et al., 1996) that the difference is due to the different methods for the determination of plasma concentration of atropine. Wurzbarger et al. utilized RIA method to detect plasma concentration of atropine. Moore et al. (1991) has studied pharmacokinetics in sheep. Unfortunately, they did not report the weight normalized clearance and other pharmacokinetic parameters.

Integration of Pharmacokinetics and Pharmacodynamics.

Despite the clinical use of antimuscarinic agents for many years, relatively little data is available on the relationship between their pharmacokinetic and pharmacologic activity. It is well known that antimuscarinic agents have numerous pharmacological activity. Integration of heart rate, saliva flow, and CNS activity with atropine pharmacokinetics have been attempted, but not a solid PK/PD model related to these group of agents have been established.

Hinderling et al. (1985a; 1985b) applied a data analysis approach, which

simultaneously fitted the pharmacokinetics and pharmacodynamic data to an integrated kinetic-dynamic model employing the digital computer program TOPFIT. It was assumed that atropine was pharmacologically active, but any of its metabolite was inactive.

The kinetic part of the model consists of a linear intravenous infusion two-compartment model with central (P) and peripheral (tissue) compartment (T), with elimination from P. The dynamic part of the model comprised two effect compartments (T_{hr} and T_{sf}) which are kinetically undistinguished from the peripheral compartment (T). The intensity of the heart rate response (increase), E_{hr} , and the saliva response (decrease), E_{sf} , are proportional to the amount of drug in the peripheral compartment, T, in accordance with the empirical equation:

$$E_{hr} = (E_{max} - E_0)_{hr} * \left(\frac{T^\gamma}{T^\gamma + T_{(50)}^\gamma} \right)_{hr} + E_{0,hr}$$

$$E_{sf} = E_{0,sf} * \left(\frac{T_{(50)}^\gamma}{T^\gamma + T_{(50)}^\gamma} \right)_{sf}$$

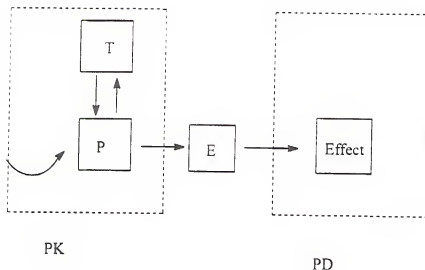
where E_{max} , $T_{(50)}$, and E_0 correspond, respectively, to the true maximum effect, the amount of drug in the peripheral compartment evoking a one-half maximum response, and the baseline effect. γ is the Hill coefficient.

The results demonstrated that the drug effects were proportional to the amounts of atropine in the peripheral compartment. The author found that a statistically significant positive linear correlation between the mean heart rate and the mean amounts of drug in the peripheral compartment.

Ellinwood et al. (1990) designed two experiments to examine the pharmacokinetic-pharmacodynamic relationship for the central nervous system and

peripheral effect of atropine. The central nervous system tests included wheel tracking, a coordination task, digit symbol substitution, a memory-psychomotor speed task; the physiological variable was the heart rate. They found that changes in plasma atropine levels and heart rate showed positive correlation. In contrast, the CNS activity were not correlated with plasma concentration of atropine .

Scheinin et al. (1998) developed a new PK-PD modeling to elucidate the time course and concentration-effect relationship of parasympatholytic effects of three anticholinergic drug using spectral analysis of heart rate (HR) variability. The concentration of the anticholinergic agents in plasma were determined by radioreceptor assay (RRA), and the pharmacokinetics of three agents were adequately described by a two-compartment open model. A hypothetical effect compartment body model was proposed to link the pharmacokinetic and pharmacodynamic part the models (Scheme 1-3). The classical parametric approach based on a hypothetical effect compartment linked to the central compartment by a first-order process was used. The concentration of the drug in effect compartment was fitted to a sigmoid inhibitory effect model with baseline effect using the individual PK parameters as constants. Equation 1 (effect site concentration) and equation 2 (effect) were fitted together using the nonlinear regression program PCNOLIN.



Scheme 1-3. A PK/PD model linked by a hypothetical effect compartment (E). T: tissue compartment; P: central compartment.

$$C_e = \frac{D k_{e0}}{V_c} \left[\frac{(k_{21} - \alpha) e^{-\alpha t}}{(\beta - \alpha)(k_{e0} - \alpha)} + \frac{(k_{21} - \beta) e^{-\beta t}}{(\alpha - \beta)(k_{e0} - \beta)} + \frac{(k_{21} - k_{e0}) e^{-k_{e0} t}}{(\alpha - k_{e0})(\beta - k_{e0})} \right] \quad (1)$$

$$E = E_0 + \frac{E_{\max} C_e}{EC_{50} + C_e} \quad (2)$$

where C_e is the drug concentration at the effect-site. D is the dose. k_{e0} is the equilibration rate constant. V_c , k_{21} , α , and β are the modeled PK parameters. t is time after injection. E is the effect, E_0 is the baseline. E_{\max} is the maximal effect. EC_{50} is the concentration at the 50% of E_{\max} , and γ is the sigmoidicity (Hill) factor.

Anticholinergics as Mydriatic Agents

Autonomic Systems in Eye

The iris-ciliary body is composed of smooth muscles which are innervated by the autonomic nervous system. The iris is a forward extension of the choroid and arises from the anterior face of the ciliary body. The pupil forms a central aperture in the iris. Two different types of muscle layers are contained in the iris. These are dilator and sphincter muscles. The dilator muscles receive sympathetic innervation. An increase in activity of these muscles produces mydriasis. The sphincter muscles receive parasympathetic innervation. An increase in activity of these muscles produces miosis. The ciliary muscle is composed of smooth muscle units which receive parasympathetic innervation. Stimulation of these muscles causes contraction, which in turn results in a lessening of the tension on the suspensory ligaments which causes lens to change shape so that it becomes more convex and allows near objects to be brought into focus at the retina.

Clinical uses of mydriatics include their diagnostic use for accurate examination of retina and optic disc and in the treatment of acute iritis, iridocyclitis, and keratitis (Moroi and Lichter, 1996). Complete cycloplegia may be useful in certain clinical use.

Anticholinergics as Mydriatic Agents

Atropine, scopolamine, homatropine, cyclopentolate and tropicamide represent the anticholinergic drugs currently marketed as mydriatic-cycloplegic agents. These agents are used in ophthalmic procedures for the examination of the interior of the eyes.

However, when these agents applied as eye drops into the eye, side effects resulted from drainage of the agents into systemic circulation could be serious, sometimes dangerous. At least 6 deaths have been attributed to the ocular administration of atropine (Frunfelder, 1989). Various psychic disturbances have been reported to the application of atropine (Wright, 1992), scopolamine (Birkhimer et al., 1984), and cyclopentolate (Khurana et al., 1988) as mydriatic/cycloplegic agents. Even for the relatively safe agent tropicamide, a myasthenia gravis-like syndrome has been described after topical application (Meyer et al., 1992). Anticholinergic eye drops have long been regarded as important household poison because accidental ingestion of small quantities can produce a severe atropine-psychosis and are particularly dangerous in children (Westfall, 1983). Another drawback of these agents are their long duration of action. In clinical setting, the time for the patients to recover from the myriatic/cycloplegic action of this agents range from six hours (tropicamide) and several days (atropine) (Brown and Taylor, 1996). Because of the prolonged recovery time from the effects of diagnostic mydriasis, many patients are reluctant to undergo diagnostic mydriasis during an office visit (Steinmann et al., 1987).

It is imperative that a new class of mydriatic agents, which are safer and short-acting, are needed to be developed.

CHAPTER 2

RESEARCH DESIGN AND SPECIFIC AIMS

Objective

The main objective of the work described in this dissertation was to develop a novel soft anticholinergics. This new class of soft anticholinergics are expected to fulfill the requirement of the soft drug: potent agents with controllable and predictable metabolism. This new class of agents are expected to be locally active but systemically inactive. They could be useful as mydriatic agents or safe antiperspirants.

Design of soft anticholinergics based on N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acid

N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acid have the general structure as I (Figure 2-1). Originally, they are synthesized as bronchodilator agents (Turbanti et al., 1992). Pharmacological studies have demonstrated that some of the compounds showing high anticholinergic activity (Turbanti et al., 1992). Receptor binding and functional studies indicated that one of the compounds, (\pm) -3[[[(2-phenylcyclohexene-1-yl)carbonyl]oxy]-8,8-diethyl-8-azoniabicyclo[3.2.1]octane iodide, is the potent and selective antagonist for the M_3 -receptor subtype (D' Agostino et al., 1994).

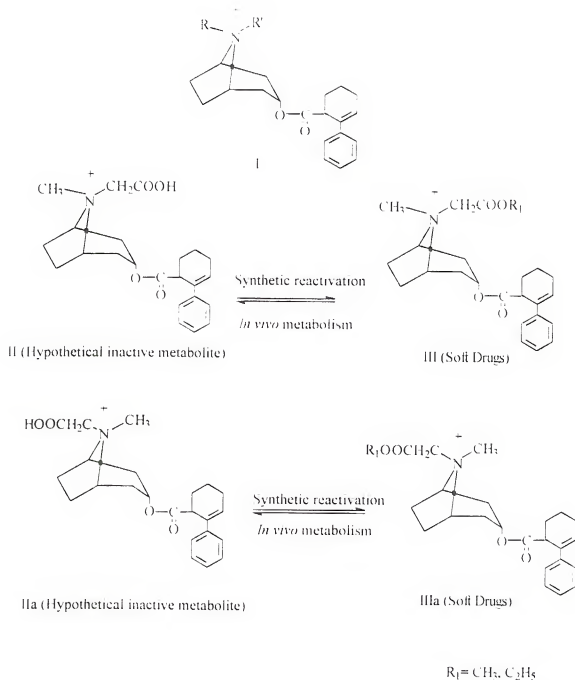


Figure 2-1. Design of a new class of soft anticholinergics based on inactive metabolite.

These characteristics prompt us to investigate the possibility to design safe antiperspirant and/or mydriatic agents based on N-alkyl-nortropine esters of 2-phenyl-cyclohexenic acids.

It is expected that, like all other anticholinergics, prolonged utilization of N-alkyl-nor-tropine esters of 2-phenyl-cyclohexenic acids as antiperspirants will result in unwanted systemic side effects (see Introduction).

It is desirable to design soft anticholinergics based on N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acids. The inactive metabolite approach for the design of soft drugs advanced by Bodor (1984) and adopted by Hammer et al.(1988), Kumar et al. (1993a; 1993b) is applicable to the design of soft drug based on N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acids. A hypothetical metabolite II (Figure 2-1) is chosen as lead metabolite for the design of the soft drugs. Even though II has not been detected as the metabolite of N-alkyl-nor-tropine esters of 2-phenyl-2-cyclohexenic acids, it is a logical choice as a lead compound, since it is the highest oxidized state of the axial group of N-alkyl substituent, which will ensure to "avoid oxidation during the *in vivo* metabolism as much as possible" (Bodor, 1990). The designed soft drugs are expected to hydrolyze rapidly to III *in vivo*. Another hypothetical metabolite IIa is an isomer of II. IIa has the axial instead of equatorial position of the R group. The smaller the size at equatorial N-substituent of N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acids, the more potent the compound is (Turbanti et al., 1992). It is expected the soft drug based on IIa as the lead will be less potent than II. The hypothetical metabolites are expected to be highly polar and ionized at physiological pH, and thus be subject to facile elimination from the systemic circulation either directly

or after conjugation. Strong nucleophilic groups are shown to be present at the muscarinic receptor site (Sokolovsky, 1984). There is a possibility that the carboxylate metabolites (I or IIa) that results from the hydrolysis of the soft drugs will have an unfavorable interaction with the receptor site are less active than the original soft drug. The hydrolysis is expected to be facile in the skin due to the abundant presence of non-specific esterase in skin. Thus a shorter local action with potentially reduced systemic side effects can be visualized with these soft drugs.

Specific Aims

The aim of these studies is the development of a new class of soft anticholinergics. This new class of soft anticholinergics are expected to be active locally at the site of application but are hydrolyzed in a facile manner in the systemic circulation to an inactive polar metabolite. The therapeutic index of this new class of soft anticholinergics should be increased. The experimental protocol is as follows:

1. Synthesis of two series of soft anticholinergic agents.
2. Development of a suitable analysis system to evaluate the stability and the metabolic pathways of the soft drugs synthesized.
3. Evaluation of *in vitro* stability in various biological media.
4. Evaluation of *in vitro* pharmacodynamic activity by receptor binding studies.
5. Evaluation of *in vivo* activity in suitable animal models.
6. *In vivo* pharmacokinetic studies of selected soft anticholinergics.

CHAPTER 3 MATERIALS AND METHODS

Materials

All chemicals used were reagent grade. Pirenzepine and (\pm)-p-fluoro-hexahydro-sila-difenidol hydrochloride (p-F-HHSiD) were obtained from Research Biochemical International (Natick, Massachusetts). N-[3 H]-Methyl- scopolamine was obtained from Dupont NEN Research (Boston, MA). Atropine, scopolamine, and propantheline were from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were from Aldrich Chemical Company (Milwaukee, Wisconsin). Scintiverse BD and other solvents were from Fisher Scientific Co. (Pittsburgh, PA). All melting points were recorded using Fisher-Johns melting point apparatus and are uncorrected. NMR data were recorded with Varian 300 NMR spectrometer and are reported in parts per million (δ) relative to tetramethylsilane. All compounds were dissolved in $CDCl_3$. The elemental analysis were carried out at Atlantic Microlab, Inc.(Atlanta, Ga). Thin layer chromatography was carried out using EM Science DC-Plastic foil plate coated to a thickness of 0.2 mm with silica gel 60 containing florescent (254) indicator. Column chromatography was performed with silica gel (70-230) with appropriate mobile phase. All the animal studies were conducted in accordance with the guidelines set forth in the Declaration of Helsinki

and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). The following strains of animals were used in the studies: (1) male New Zealand albino rabbit weight 3 kg. and (2) male Sprague Dawley rats weighting 250-300 gms.

Methods

Synthesis of soft Anticholinergics Based on Propyl 2-Phenyl-2-Cyclohexen-1-Carboxylate (9a, 9b, 13a, and 13b) (Figure 3-1 and Figure 3-2)

Protection of ethyl 2-oxo-cyclohexanecarboxylate (1)

A mixture of ethyl 2-oxocyclohexanecarboxylate (12 g, 0.0705 mole), 1,2 diethanol (10.9 g, 0.178 mole), PTSA (205 mg, 1.06 mmole) in 300 ml of benzene was refluxed with stirring for 20 hours. The mixture was diluted with Et₂O, the organic layer was washed with saturated NaHCO₃ (2x 100 ml.), brine (2x100 ml.) and dried over Na₂SO₄. Ether was removed under reduced pressure to afford an oily crude product, which was distilled at 70°-75°C (0.35 mm Hg) to give ethyl 2-ketal-cyclohexanecarboxylate.

Reduction of ethyl 2-ketalcyclohexanecarboxylate (2)

A mixture of 200 ml of absolute ether and 100 ml of 1.0 M LiAlH₄ in THF was stirred under argon in ice bath for 30 minutes. To this mixture a solution of **2** (17.5g, 0.0816 mole) in Et₂O (100 ml) was added dropwise with stirring. When the addition was

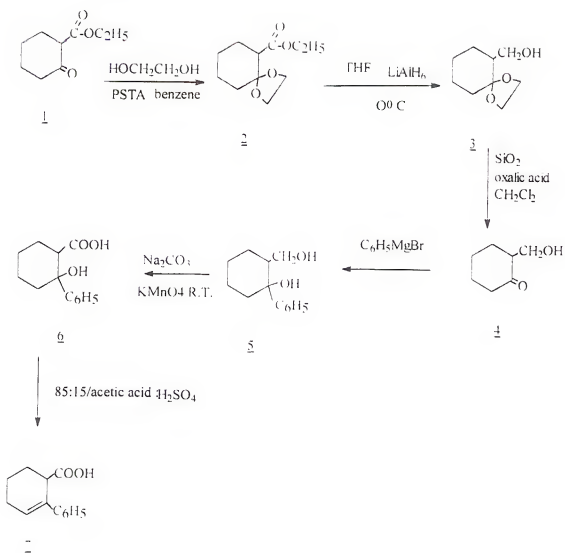


Figure 3-1. Synthesis of 2-phenyl-cyclohexenic acid.

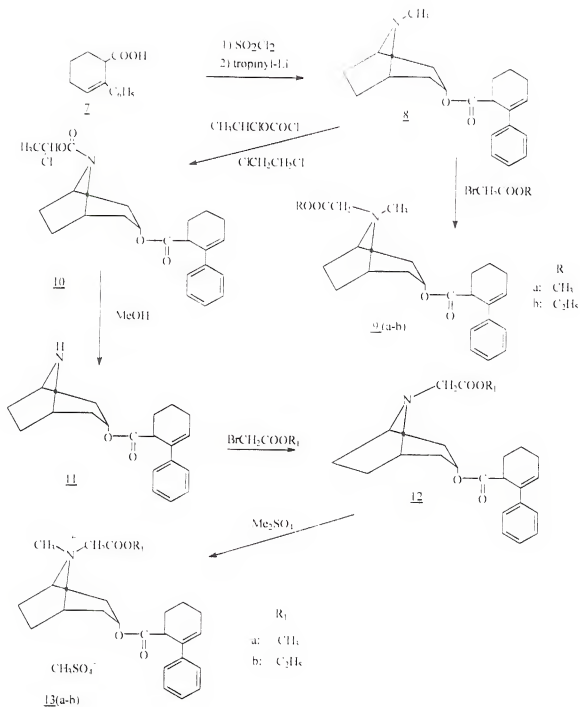


Figure 3-2. Synthesis of 9a, 9b, 13a, and 13b.

ended, the ice bath was removed, the mixture was allowed to warm to r.t. and stirred for 17 hour. To the stirred mixture was added slowly water (3.8 ml), 15% NaOH (14ml), water (14 ml), and anhydrous Na_2SO_4 (60 g) in that order. The mixture was filtered and the solvent removed under reduced pressure to give colorless liquid, which was distilled at 65 - 70°C (0.2 mm Hg) to afford 2-hydroxymethylcyclohexane ethylene ketal.

Deketalization of 2-hydroxymethylcyclohexane ethylene ketal (3)

To 105 g of SiO_2 in 700 ml of CH_2Cl_2 was added dropwise 1.5 g of oxalic acid in 14 ml of H_2O with continuous stirring until it mixed in about 15 minutes. To the above mixture, 16 g of 2-hydroxymethylcyclohexane ethylene acetal (3) was added with continuous stirring at r.t. for 3 days. Distillation of the product at 70°C (0.3mm Hg) to give 2-hydroxymethylcyclohexanone (4)

Grignard reaction with 2-hydroxymethylcyclohexenone (4)

To a well-stirred cold mixture of 2.0 moles of anhydrous phenylmagnesium bromide in 750 ml of anhydrous ether under argon, was added dropwise 64.09 g (0.5 mole) of 2-hydroxymethylcyclohexenone (4) in 100 ml of dry absolute ether with stirring on ice bath. The addition required one hour; stirred with cooling in ice was continued for an additional one-half hour. During this time a crystalline complex separated. The remaining mixture was then decomposed by pouring onto ice-cold saturated ammonium chloride solution. The ether extracts (crystalline complex) were dried over sodium sulfate and concentrated under reduced pressure to give crude product, which was distilled at 126-128°C (0.1-0.12 mm Hg) to afford a thick oil. The oily product was brought to

crystallize in hexane (45 ml) and ether (5 ml) to generate 1-phenyl-2-hydroxymethyl-cyclohexanol.

Oxidizing 1-phenyl-2-hydroxymethylcyclohexanol (5)

To a solution of potassium permanganate (10 g, 0.032 mole) and dry sodium carbonate (5 g) in 500 ml of water, was added 5 g (0.024 mole) of finely powered 1-phenyl-2-hydroxymethylcyclohexanol. The suspension was stirred at the room temperature for 21.5 hours. The manganese dioxide was filtered off and the filtrate was decolorized with sodium bisulfite and then acidified with concentrated hydrochloric acid. The above mixture was filtered to yield 2-phenyl-2-hydroxycyclohexane-1-carboxylic acid (6)

Dehydration of 2-hydroxy-2-phenylcyclohexane-1-carboxylic acid (6)

2-Hydroxy-2-phenylcyclohexane-1-carboxylic acid (6, 1 g, 4.5 m mole) was added to a mixture of sulfuric acid (1.5 ml) and acetic acid (8.5 ml) with stirring at room temperature. The mixture was stirred for another 15 minutes to dissolve all solids then poured into 100 ml of ice water with stirring. After white crystallized solids appeared, the mixture was continuously stirred for another 30 minutes. After stored in -20°C refrigerator overnight, the above mixture was filtered to give 2-phenyl-2-cyclohexen-1-carboxylic acid (7)

Synthesis of 2-phenyl-2-cyclohexene-1-carboxylic chloride

To 2-phenyl-2-cyclohexen-1-carboxylic acid (7, 1g, 4.93 m mole) in 15 ml of absolute ethyl ether with one drop of DMF, thionyl chloride (0.65g, 5.43 m mole) was

added. The above solution was refluxed under argon for 2 hours. Solvent and excess ether was removed under reduced pressure. Three portion of 10 ml of dry benzene were added and removed under reduced pressure to give reddish brown oily 2-phenyl-2-cyclohexene-1-carboxylic chloride.

Synthesis of tropyl 2-phenyl-2-cyclohexen-1-carboxylate (8)

To tropine (1.32 g 9.38 m mole) in 10 ml of anhydrous THF, was added by syringe butyl lithium (4.69 ml of 2 M hexane solution, 9.38 m mole) at 0°C under argon atmosphere. Then the mixture was stirred at room temperature for 30 minutes. To the above solution was added 2-phenyl-2-cyclohexene-1-carboxylic chloride in THF at 0°C. The mixture was stirred at room temperature for 19 hours. The above residue from evaporation of solvent was acidified by adding 1 N HCl until pH 2 and extracted with ether twice. Aqueous phase was basified with NaHCO₃ until pH 8 and extracted with ether again. Organic layer was separated. Evaporated to give yellowish oil, which was purified by flash chromatography on a silica gel (methanol: NH₄OH=100:2.5) to yield pure **8**.

Synthesis of 2-phenyl-2-cyclohexen-1-carb-Nβ-methoxycarbonylmethyltropinium bromide (9a) and 2-phenyl-2-cyclohexen-1-carboxyly-Nβ-methoxycarbonylmethyltropinium bromide (9b).

To **8** (6.14 m mole) of tropyl 2-phenyl-2-cyclohexen-1-carboxylate (**8**) in 20 ml anhydrous acetonitrile, 15.36 m mole of methyl bromoacetate or ethyl bromoacetate was added. The above mixture was stirred under argon for 19 hours. Evaporation of

acetonitrile to generate an oily substance, which was further purified by precipitate to give pure 9a or 9b.

Synthesis of nortropyl 2-phenyl-2-cyclohexen-1-carboxylate (11)

To 1 g (3.07 mmole) of 8 in 10 ml of 1,2-dichloroethane at 0 °C, 1.09g (7.63 mmole) of 1-chloroethyl chloroformate in 5 ml of 1,2-dichloroethane was added dropwisely. The mixture then refluxed for 1 hour. Residue from evaporation of solvent of the reaction mixture was then refluxed in methanol for 45 minutes. Removal of methanol under reduced pressure gave 11.

Synthesis of methoxycarbonylmethylnortropyl 2-phenyl-2-cyclohexen-1-carboxylate (12a) and ethoxycarbonylmethylnortropyl 2-phenyl-2-cyclohexen-1-carboxylate (12b)

To well stirred compound 11 (1 g, 3.21 mmole) in 20 ml of N,N-dimethyl formamide (DMF) with 1 g of K_2CO_3 , was added 3.21 mmole of methyl bromoacetate or ethyl bromoacetate. The mixture was stirred under argon for 20 hours. Then the DMF was removed under reduced pressure. The residue was added with 5 ml saturated $NaHCO_3$ solution, which was then extracted 3 times with absolute ethyl ether to give crude 12. 12 was further purified by flash chromatography on ethyl acetate to give pure 12a or 12b.

Synthesis of 2-Phenyl-2-cyclohexen-1-carboxyl -N α -methoxycarbonylmethyltropinium methylsulfate (13a) and 2-phenyl-2-cyclohexen-1-carboxyl -N α -ethoxycarbonylmethyltropinium methylsulfate (13b).

To compound 12 (a or b, 2.50 mmole) in 10 ml of anhydrous acetonitrile was added dimethyl sulfate (0.788 g, 6.25 mmole). The mixture was stirred at room temperature for 15 hours and then acetonitrile was removed under reduced pressure. The residue was purified by adding methylene chloride-dissolved mixture dropwise into ethyl ether to give precipitated 13a and 13b.

Synthesis of Phenylcyclopentyl Acid: Series of New Class of Soft Anticholinergics (15a,15b,18a, and 18b) (Figure 3-3).

Synthesis of tropyl α -phenylcyclopentaneacetate (14)

To α -phenylcyclopentaneacetic acid (2 g, 9.79mmol) in 15 ml of absolute ethyl ether, 1 drop of N, N-dimethylformamide and thionyl chloride (1.28g, 10.77 mmol) were added at room temperature. The mixture was refluxed for 2 hours. Then the ethyl ether was removed under reduced pressure to give oily α -phenylcyclopentaneacetyl chloride.

To tropine hydrochloride salt (10.77 mmol, 1.913g) in 15 ml of nitromethane, was added the above oily α -phenylcyclopentaneacetyl chloride in 5 ml of nitromethane. Then the mixture refluxed for 24 hours. Removal of nitromethane to give oily substance, which was basified with NaHCO_3 . The mixture was extracted 3 times with ethyl ether to give crude tropyl α -phenylcyclopentaneacetate, which was further purified by flash silica chromatography on silica gel (Methanol : NH_4OH = 100:2.5) to give pure tropyl α -phenylcyclopentaneacetate (14)

Synthesis of Phenylcyclopentyl-N β -methoxycarbonylmethyltropinium bromide (15a) and Phenylcyclopentyl-N β -ethoxycarbonylmethyltropinium bromide (15b).

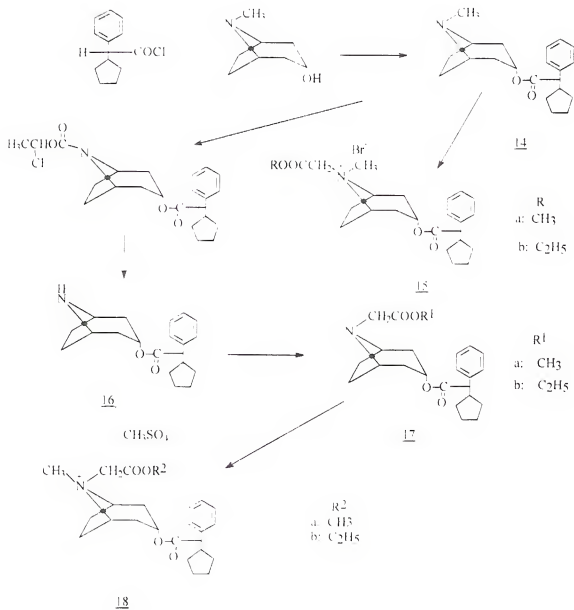


Figure 3-3. Synthesis of 15a, 15b, 18a, and 18b.

To 2 g (6.10 mmol) tropyl α -phenyleclopentaneacetate (14) in 20 ml anhydrous acetonitrile, 15.26 mmol of methyl bromoacetate or ethyl bromoacetate was added. The above mixture was stirred under argon pressure for 19 hours. Evaporation of acetonitrile to generate an oily substance, which was further purified by precipitate (methylene chloride/ethyl ether) to give pure 15a or 15b.

Synthesis of nortropyl α -phenyleclopentaneacetate (16)

To 1 g (3.05 mmole) of tropyl α -phenyleclopentaneacetate in 10 ml of 1,2-dichloroethane at 0 °C, 1.09 g (7.63 mmole) of 1-chloroethyl chloroformate in 5 ml of 1,2-dichloroethane was added dropwisely. The mixture then refluxed for 1 hour. Evaporation of the reaction mixture in vacuum to give oily residue, which was refluxed in methanol for 45 minutes. Removal of methanol under reduced pressure gave 16.

Methoxycarbonylmethylnortropyl-N(α -phenyleclopentaneacetate (17a) and Ethoxycarbonylmethylnortropyl-N(α -phenyleclopentaneacetate (17b))

To well stirred compound 16 (1g, 3.19 mmole) in 20ml of N,N-dimethyl formamide (DMF) with K₂CO₃ g, was added 3.19 mmole of methyl bromoacetate or ethyl bromoacetate. The mixture was stirred under argon pressure for 20 hours. Then the DMF was removed under reduced pressure. The residue was extracted 3 times with absolute ethyl ether to give 1.12 g of crude 17, which was further purified by flash chromatography on silica gel with ethyl acetate to give pure 17a (0.98 g, 79.7%) or 17b (1.01g,79.2%).

Phenylcyclopentyl-N α -methoxycarbonylmethyltropium methylsulfate (18a) and Phenylcyclopentyl-N α -ethoxycarbonylmethyltropium methylsulfate (18b)

To compound 17 (a or b, 2.50 m mole) in 10 ml of anhydrous acetonitrile was added dimethyl sulfate (0.788 g, 6.25 m mole). The mixture was stirred at room temperature for 15 hours and then acetonitrile was removed under reduced pressure. The residue was purified by adding dropwise methylene-dissolved mixture into ethyl ether to give precipitated 18a or 18b.

In vitro Pharmacodynamic Evaluation of the Activity of the Soft Anticholinergics-Receptor Binding

Methods for receptor binding studies of soft anticholinergics

Binding studies were performed with [³H]-methyl-scopolamine following the protocol from RBI Co. (Natick, Massachusetts). Binding buffer (Phosphate Buffered Saline-PBS, pH 7.4) consisted of 0.15 M NaCl, 1.5 mM KH₂PO₄ and 2.7 mM Na₂HPO₄. 10 mM NaF was added into the buffer as an esterase inhibitor. The assay mixture (1 ml) contained 100 μ l diluted membranes (receptor proteins, final concentration: m1, 25 μ g/ml; m2, 42 μ g/ml; m3, 15.9 μ g/ml; m4, 20 μ g/ml). The final concentrations of NMS for the m2-m4 binding studies were 0.5 nM, and for m1 was 1 nM. Specific binding was defined as the difference in [³H] NMS binding in the absence and presence of 1 μ M atropine. Incubation was carried out at room temperature for 60 minutes. The assay was terminated by filtration through a Whatman GF/B filter (presoaked with 0.5%

polychthylenimine). The filter was then washed three times with 10 ml ice cold binding buffer, transferred to vials and 10 ml of scintiverse liquid were added. Finally, detection was performed on a Packard 31800 liquid scintillation analyzer (Packard Instrument Inc., Downer Grove, IL).

Data analysis

Data from the binding experiment were fitted the following equation:

$\%[^3\text{H}] \text{ NMS bound} = 100 - [100x^n / k / (1 + x^n / k)]$ to obtain Hill coefficient n . Then to: $\%[^3\text{H}] \text{ NMS bound} = 100 - [100x^n / \text{IC}_{50} (1 + x^n / \text{IC}_{50})]$ to obtain IC_{50} , where x = concentration of the tested compound (in a series concentration). K_i was derived by the method of Cheng and Prusoff (1973): $\text{K}_i = \text{IC}_{50} / (1 + L / \text{K}_D)$, where L is the concentration of the radioligand, IC_{50} is the concentration of drug causing 50% inhibition of specific radioligand binding and K_D is the dissociation constant of radioligand receptor complex. Data were analyzed by a nonlinear least-squares curve fitting procedure using the program Scientist (MicroMath Inc., Salt Lake City, UT).

In vivo Pharmacodynamic Evaluation of the Compounds - Mydriatic Study

Using atropine-MeBr and tropicamide as reference compounds, the mydriatic activities of the newly synthesized soft anticholinergics were evaluated. Tropicamide ophthalmic solution (1%) was purchased from Schein Pharmaceutical Inc. (Florham Park, NJ). Healthy male New-Zealand White rabbits, each weighing about 3.0 kg, were used in

the experiments. For studying mydriatic activities, a dose of 100 μ l of each drug in water at various concentrations was administered in one eye, and the other eye was used as a control. Experiments were carried out in a light and temperature controlled room. At appropriate time intervals, pupil diameters for both eyes were recorded.

In vivo Pharmacodynamic Evaluation: Cardiac Studies of Selected Soft Anticholinergics

The following procedures were used: Antagonistic effect on carbachol induced bradycardia.

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, IN), each weighing 300 ± 30 g, were anesthetized with 50 mg/kg i.p. Na pentobarbital. Baseline electrocardiography (ECG) recordings and all drug administrations were performed after 15 minutes stabilization periods. Needle electrodes were inserted s.c. into the limbs of the anesthetized rats and were joined to a Gould 2000 recorder (Gould Inc., Cleveland, OH). Standard leads I, II, and III were recorded at a paper speed of 25 mm/sec. Recordings were taken before, during, and after the administration of any of the compounds until all basic ECG parameters returned to that of the baseline. ECG recordings were evaluated for the following parameters: PP cycle length (msec), RR cycle length (msec), heart rate (1/min) by the equation of $60000/\text{RR cycle length}$, and presence of Mobitz II type atrio-ventricular (A-V) block (2:1, 3:1, etc.). Cholinomimetics such as carbachol have four primary effects on the cardiovascular system: vasodilation, a decrease in cardiac rate (negative chronotropic effect), a decrease in the rate of

conduction in the sinoatrial (SA) and atrioventricular (AV) nodes (negative dromotropic effect), and a decrease in the force of cardiac contraction (negative inotropic effect) (Higgins et al., 1973). To evaluate the effects of carbachol, only the negative chronotropic and dromotropic effects were analyzed here. These effects of carbachol were manifested on the surface ECG as sinus bradycardia (lengthening of the PP cycle) and as a development of Mobitz II type A-V block. After analyzing the ECG recordings, both heart rate and percent changes of heart rate, as compared to that of the baseline, were plotted against time, and the effects of the different drugs on the heart rate and on the percent changes of the heart rate were characterized. Each point on the figures represents the mean \pm S.D. of three experiments. All drugs were dissolved in 0.9% NaCl (vehicle), and solutions were administered by direct injections into the jugular veins on either side of the rats. Anticholinergic drugs (such as 9a and 13a) and atropine (0.2 and 2 $\mu\text{mol/kg}$ = 0.102 and 1.02 mg/kg, in \sim 0.3 ml volume) or vehicle (\sim 0.3 ml volume) were administered at 0 time, while carbachol (5 - 8 $\mu\text{g/kg}$ = 27 - 44 pmol/kg in \sim 0.06-0.1 ml volume according to the initial individual ECG response of each rat) was injected at -5, 1, 3, 5, 10, 15, 20, 30, 45, and 60 minutes (with some exceptions). Analysis of variance followed by Duncan's test was used for statistical evaluation.

In vitro Pharmacokinetic Evaluation of the Soft Anticholinergics - Stability Studies

Analytical Method

A high performance liquid chromatographic method has been developed to assay the soft drug. The system consisted of a Spectra Physics (San Jose, CA) SP 8810 isocratic

pump, SP 8450 uv-vis detector with detection set to 254 nm, and an SP 4290 integrator. A Supercosil LC ABZ column (Supelco, Bellefonte, PA) was used, and the mobile phase (flow rate of 1.5 ml/min) consisted of acetonitrile /water (40:60), with final concentration of 0.1% octanesulfonic acid, 0.2% acetic acid, and 0.1% THF.

Stability in Biological Media

The following biological media will be used in the study: rat plasma, rat blood, and rat liver homogenate (25%). Procedure: To the biological medium (2ml), was added 10 µl of the stock solution of the compound, and the sample was mixed. The mixture was kept at 37°C while being shaken. Samples (100 µl) were withdrawn at the appropriate time intervals and immediately diluted with 200 µl of ice-cold acetonitrile to stop enzymatic reaction and vortexed. The supernatant after centrifugation was analyzed by HPLC for both the original compound and degradation products.

In vivo Pharmacokinetic Evaluation of the Soft Anticholinergics- In Vivo Pharmacokinetic Studies

Pharmacokinetic studies of soft anticholinergics

Once the assay was validated. The pharmacokinetics and metabolism studies of the compounds can be performed.

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). The soft anticholinergics were injected into tail vein, or jugular vein, over one minute, at a dose of 5, 10, 15 mg /kg and a dosing volume of 8 ml/kg. For the data

treatment (as bolus injection), the mid-time of the injection was used as 0 time. The tail vein injections were conducted very carefully to assure that no leakage occurred during the injection. Blood samples, 0.1 ml, were collected through the jugular vein at appropriate time intervals for 150 minutes. Subsequently, the urine samples were taken and animals were sacrificed by over dose of pentobarbital.

Noncompartmental and compartmental pharmacokinetic analysis

For noncompartmental analysis, the area under the curve, AUC, of the blood concentration versus time were calculated using the trapezoidal rule, and the area from the last measurement, C_t , to infinity was calculated as C_t/β , where β was the terminal disposition rate constant. The total body clearance, CL_{tot} , was calculated as $Dose/AUC$. Mean resident time, MRT, was calculated as $AUMC/AUC$, where AUMC, the area under the first moment curve, was calculated using the trapezoidal rule from the curve of "blood concentration x time vs time", and the area from the last time point, t , to infinity was calculated as $C_t/\beta + C_t/\beta^2$. The volume of distribution at the steady state, Vd_{ss} , was calculated as CL_{tot} multiplied by MRT. For compartmental analysis, a pharmacokinetics analysis program, PK-Analyst (Micromath, Salt Lake City, UT) was used to assist analyses.

CHAPTER 4 RESULTS AND DISCUSSION

Synthesis

The physical and spectral characteristics of the compounds synthesized are given below :

Ethyl 2-ketal-cyclohexanecarboxylate (2, 3 g, 95 %). Viscous liquid. ^1H NMR (CDCl_3): 1.21(3H, t, CH_3CH_2), 1.4-2.0 (8H, overlapping, cyclohexyl H) , 2.61-2.70 (1 H, dd, CHCO_2), 3.90-3.98 (4H, m, $\text{OCH}_2\text{CH}_2\text{O}$), 4.25 (2H, q, CH_3CH_2) ppm.

2-Hydroxymethylcyclohexane ethylene ketal (3, 13.2g, 0.076mole, 93 %). Liquid. ^1H NMR (CDCl_3): 1.28-1.90 (8H, overlapping, cyclohexanyl), 2.88 (1H, br, CHCO_2), 3.51-3.56 (1H, m, CH_2OH), 3.66 (1H, m, CH_2OH), 3.98-4.01 (4H, m, $\text{OCH}_2\text{CH}_2\text{O}$) ppm.

2-Hydroxymethylcyclohexanone (4, 5.45g, 0.418 mole, 45 %). Liquid. ^1H NMR (CDCl_3): 1.45-2.55 (8H, m, cyclohexanyl), 2.79 (1H, b, CHCO_2), 3.57-3.90 (2H, 2xm, CH_2OH) ppm.

1-Phenyl-2-hydroxymethyl-cyclohexanol (5, 27.0 g, 25 %). ^1H NMR (CDCl_3): 1.40-2.10 (9H,m, Cyclohexanyl), 3.40-3.55 (2H, m CH_2OH), 7.20-7.50 (5H, m, ph) ppm.

2-Phenyl-2-cyclohexen-1-carboxylic acid (7, 0.75 g, 83 %), ^1H NMR (CDCl_3): 1.71-2.56 (6H, m, 3CH_2 , cyclohexanyl), 3.69 (1H, b, CHCO_2), 6.22 (1H, t, cyclohexene's 3-H), 7.20-7.33 (5H, m, ph) ppm.

Tropyl 2-phenyl-2-cyclohexen-1-carboxylate (8, 0.49 g, 30%), Liquid. ^1H NMR (CDCl_3): 1.20-2.27 (14H, m, 3CH_2 cyclohexenyl, 4CH_2 , tropine), 2.26 (3H, s, NCH_3), 2.98-3.10 (2H, brd, tropine's 1,5-H), 3.70 (1H, s, CHCO_2), 4.87 (1H, CHO), 6.22 (1H, s, cyclohexene's 3-H), 7.25-7.34 (5H, m, ph) ppm.

2-Phenyl-2-cyclohexen-1-carboxyl- N_{10} -methoxycarbonylmethyltropinium bromide (9a) and 2-phenyl-2-cyclohexen-1-carboxyl- N_{10} -ethoxycarbonylmethyltropinium bromide (9b).

9a (2.4 g, 81%), M.P. 170-171°C, ^1H NMR (CDCl_3): 1.26-2.28, 2.63-2.69 (14H, m, 3CH_2 cyclohexenyl, 4CH_2 , tropine), 3.57 (3H, s, NCH_3), 3.78 (4H, s, OCH_3 and 1-H of cyclohexenyl), 4.59-4.69-4.62 (2H, br d, tropyl's 1,5-H), 4.84 (2H, NCH_2), 5.05 (1H, t, CHO), 6.28 (1H, t, 2-H of cyclohexenyl), 7.23-7.33 (5H, m, Ph) ppm. Elemental analysis: calculated/found: C, 60.25/60.06; H, 6.74/6.81; N, 2.93/2.87.

9b (2.2 g, 73%), M.P. 186-187°C, ^1H NMR (CDCl_3): 1.26-2.60 (14H, m, 3CH_2 cyclohexenyl, 4CH_2 , tropine), 1.31 (3H, t, CH_2CH_3), 3.37 (3H, s, NCH_3), 3.76 (1H, b, 1-H of cyclohexenyl), 4.22 (2H, q, CH_2CH_3), 4.58, 4.67 (2H, br d, tropyl's 1,5-H), 4.82 (2H, NCH_2), 4.95 (1H, t, CHO), 6.22 (1H, t, 2-H of cyclohexenyl), 7.20-7.31 (5H, m, Ph) ppm. Elemental analysis: calculated/found: C, 60.97/61.10; H, 6.55/6.96; N, 2.84/2.79.

Nortropyl 2-phenyl-2-cyclohexen-1-carboxylate (11)

11 (0.93, 97%). ¹H NMR (CDCl₃): 1.46-2.25, 2.66-2.90 (14H, m, 3CH₂ cyclohexenyl, 4CH₂, tropine), 3.71 (1H, s, 1-H of cyclohexenyl), 3.80-3.85 (2H, br d, tropine's 1,5-H), 4.95 (1H, t, CHO), 6.23 (1H, t, cyclohexene's 3-H), 7.23-7.36 (5H, m, Ph) ppm.

Methoxycarbonylmethylnortropyl 2-phenyl-2-cyclohexen-1-carboxylate

(12a) and (±)-ethoxycarbonylmethylnortropyl 2-phenyl-2-cyclohexen-1-carboxylate
(12b)

12a (0.95, 75%). ¹H NMR (CDCl₃) : 1.20-2.20 (14H, m, 3CH₂ cyclohexenyl, 4CH₂, tropine), 3.01-3.06 (2H, br d, tropyl's 1,5-H), 3.06 (2H, s, NH₂) 3.63 (4H, s, OCH₃ and 1-H of cyclohexenyl), 4.82 (1H, t, OCH), 7.12-7.28 (5H, m, Ph) ppm.

12b (0.97, 76%). ¹H NMR (CDCl₃): 1.23-2.27 (14H, m, 3CH₂ cyclohexenyl, 4CH₂, tropine), 1.25 (3H, t, CH₃), 3.01-3.15 (2H, br d, tropyl's 1,5-H), 3.72 (1H, t, 1-H of cyclohexenyl), 4.15-4.17 (2H, q, CH₂CH₃), 4.89 (1H, m, OCH), 7.25-7.35 (5H, m, Ph) ppm.

2-Phenyl-2-cyclohexen-1-carboxyl-Nα-methoxycarbonylmethyltropinium methylsulfate

(13a) and 2-phenyl-2-cyclohexen-1-carboxyl-Nα-ethoxycarbonylmethyltropinium
methylsulfate (13b).

13a (1.15g, 90%). M.P.150-151°C. ¹H NMR (CDCl₃): 1.25-2.70 (14H, m, 3CH₂ cyclohexenyl, 4CH₂, tropine), 3.18 (3H, s, NCH₃), 3.66 (3H, s, CH₃SO₄), 3.71(1H, b, 1-H of cyclohexenyl), 3.77 (3H, s, OCH₃), 4.35-4.45 (2H, br d, tropyl's 1,5-H), 4.70 (2H, s, NCH₂), 5.03 (1H, t, OCH), 7.26-7.31 (5H, m, Ph) ppm. Elemental analysis: calculated/found; C, 58.92/58.97; H, 6.92/6.86; N, 2.75/2.45.

13b (1.19g, 91 %), M.P.169-170°C. ^1H NMR (CDCl_3): 1.25-2.60 (14H, m, 3CH_2 cyclohexenyl, 4CH_2 , tropine), 3.24(3H, s, NCH_3), 3.72 (4H,s, CH_3SO_4 and 1-H of cyclohexenyl), 4.24 (2H, q, $\text{CH}_2\text{CH}_2\text{O}$), 4.40-4.54 (2H, br d, trotyl's 1,5-H), 4.60 (2H,s, NCH_2), 5.01 (1H, m, OCH), 6.30 (1H, t, cyclohexenyl 1-H) , 7.21-7.40 (5H, m, Ph) ppm. Elemental analysis: calculated/found: C, 58.82/58.77; H, 5.15/7.08; N, 2.63/2.60.

Trotyl α -phenylcyclopentaneacetate (14)

(14, 2.5 g, 78%), ^1H NMR (CDCl_3): 0.9-2.1 |16H, 4xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7 -H], 2.23(3H, s, CH_3N), 2.5-2.64(1H, m, CH , Pentyl 1-H), 2.90, 3.15(2H, br d, trotyl's 1,5- H), 3.25 (1H, d, PhCH), 4.96 (1H, t, CO_2CH), 7.29-7.36(5H, m, Ph) ppm.

Phenylcyclopentyl-N β -methoxycarbonylmethyltropinim bromide (15a) and phenylcyclopentyl-N β -ethoxycarbonylmethyltropinim bromide(15b).

15a (2.2 g , 80%), M.P.178-179°C. ^1H NMR(CDCl_3): 1.00,1.20,1.40-1.82, 1.90-2.37 |16H, m, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2, 4, 6, 7 -H], 2.71, 2.78[1H, 2xbr, $(\text{CH}_2\text{CH}_2)_2\text{CH}$], 3.24(1H, d, PhCH), 3.60(3H, s, NCH_3), 3.78(3H, s, CO_2CH_3), 4.62, 4.84(2H, 2xbr., trotyl's 1,5-H), 4.80, 4.92(2H, 2d, CH_2CO_2), 5.12(1H, t, CO_2CH), 7.24-7.30(5H, m, Ar-H) ppm. Elemental analysis: calculated/ found:

15b (2.3g, 82 %), M.P. 181-182°C. ^1H NMR(CDCl_3): 1.00,1.20,1.40-1.82, 1.90-2.37 |16H, m, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2, 4, 6, 7 -H], 1.28 (3H, t, CH_3CH_2), 2.71, 2.78[1H, 2xbr, $(\text{CH}_2\text{CH}_2)_2\text{CH}$], 3.24(1H, d, PhCH), 3.60(3H, s, NCH_3), 4.22(3H, q, $\text{CH}_3\text{CH}_2\text{CO}_2$), 4.62, 4.84(2H, 2xbr., trotyl's 1,5-H), 4.7, 4.82(2H, 2d, $\text{CO}_2\text{CH}_2\text{N}$), 5.12(1H, t, CO_2CH), 7.24-7.30(5H, m, Ph) ppm. Elemental analysis: calculated/found: C, 60.73/60.47; H, 7.34/7.36; N, 2.83/2.86.

Notrotyl α -phenylcyclopentaneacetate (16)

(0.95 g, 99%), ^1H NMR (CDCl_3): 0.9-2.1 [16H, 5xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7-H], 2.51-2.53 (1H, 2br, CH. Pentyl 1-H), 3.25 (1H, d, PhCH), 3.80, 3.95 (2H, br d, trotyl's 1,5-H), 5.12 (1H, t, CO_2CH), 7.29-7.36 (5H, m, Ph) ppm.

Methoxycarbonylmethylnortrotyl- α -phenylcyclopentaneacetate (17a) and ethoxycarbonylmethylnortrotyl- α -phenylcyclopentaneacetate (17b)

17a (0.98 g, 79.7%) . ^1H NMR (CDCl_3): 0.9-2.1 [16H, 5xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7-H], 2.5-2.7 [1H, m, $(\text{CH}_2\text{CH}_2)_2\text{CH}$], 3.1, 3.2 (2H, brd, trotyl's 1,5-H), 3.15 (2H, s, NCH_2), 3.23 (1H, d, PhCH) , 3.70 (3H, s, OCH_3), 4.95 (1H, m, CO_2CH), 7.20-7.40 (5H, m, Ph).

17b (1.01g, 79.2%) . ^1H NMR (CDCl_3): 0.9-2.1 [16H, 5xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7-H], 1.25 (3H, s, CH_3), 2.5-2.7 [1H, m, $(\text{CH}_2\text{CH}_2)_2\text{CH}$], 3.1-3.2 (2H, br d, trotyl's 1,5-H), 3.15 (2H, s, NCH_2), 3.23 (1H, d, PhCH) , 4.18 (2H, q, OCH_2CH_3), 5.01 (1H, m, OCH), 7.20-7.40 (5H, m, Ph) ppm.

(\pm)Phenylcyclopentyl-N(α -methoxycarbonylmethyltrotyl) methylsulfate (18a) and phenylcyclopentyl-N(α -ethoxycarbonylmethyltrotyl) methylsulfate (18b).

18a (1.13g, 88.3%). M.P. 159-160.5 $^\circ\text{C}$. ^1H NMR (CDCl_3): 0.9-2.1 [16H, 5Xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7-H], 2.5-2.7 [1H, m, $(\text{CH}_2\text{CH}_2)_2\text{CH}$], 3.21 (3H, s, NCH_3), 3.23 (1H, d, PhCH) , 3.64 (3H, s, CH_3SO_4), 3.80 (3H, s, OCH_3), 4.25-4.57 (2H, br d, trotyl's 1,5-H), 4.81 (2H, brd, NCH_2), 5.15 (1H, t, CO_2CH), 7.20-7.40 (5H, m, Ph) ppm. Elemental analysis: calculated/ found: C, 58.68/ 58.85; H, 7.28/7.29; N, 2.73/ 2.71.

18b (1.17g, 89.0%) .M.P. 153-154 $^\circ\text{C}$. ^1H NMR (CDCl_3): 0.9-2.1 [16H, 5Xm, $(\text{CH}_2\text{CH}_2)_2\text{CH}$ and trotyl's 2,4,6,7-H], 1.20 (3H, s, CH_3), 2.5-2.7 [1H, m,

(CH₂CH₂)₂CH], 3.19(3H, s, NCH₃), 3.23 (1H, d, PhCH), 3.70 (3H, s, CH₃SO₄), 4.22 (2H, q, OCH₂CH₃), 4.40, 4.53(2H, br d, tropyli's 1,5- H), 4.65 (2H, m, NCH₂), 5.15 (1H, t, CO₂CH), 7.20-7.40 (5H, m, Ph) ppm. Elemental analysis: calculated/ found: C, 59.41/59.39, H, 7.48/7.54, N, 2.67/2.67.

Pharmacodynamic Evaluation

In Vitro Activity--- Receptor Binding Studies.

1. Receptor binding studies of reference compounds and existing soft anticholinergics.

One goal of this research was to evaluate the soft anticholinergics made in the Center for Drug Discovery at the University of Florida during the past 20 years for the development of safer antiperspirants and mydriatics. In order to establish and validate the receptor binding methods, several reference compounds were tested. The pK_i values of these compounds were listed in Table 4-1. The binding data of existing and some newly synthesized soft anticholinergics were listed in Table 4-2. For the comparison, PA₂ values were also listed in the same tables.

Table 4-1. Binding parameters of reference compounds at four muscarinic receptor subtypes. The affinity estimates were derived from [3 H]NMS displacement experiments and represented the mean (\pm S.E.M, n=3-5) for the negative logarithm of K_i . The Hill coefficients are given in parentheses.

Reference	Muscarinic Subtypes Receptor				pA2
Compound	m1	m2	m3	m4	
Atropine	9.44 \pm 0.05 (1.00 \pm 0.04)	8.96 \pm 0.06 0.97 \pm 0.03	9.14 \pm 0.04 1.11 \pm 0.02	8.96 \pm 0.10 1.00 \pm 0.03	8.95 ^a
Scopolamine	8.96 \pm 0.036 (0.99 \pm 0.03)	8.66 \pm 0.05 (1.02 \pm 0.02)	9.47 \pm 0.07 (1.02 \pm 0.01)	9.47 \pm 0.07 (1.11 \pm 0.01)	9.50
Pirenzepine	8.35 \pm 0.04 (1.08 \pm 0.03)	6.02 \pm 0.04 (0.95 \pm 0.02)	6.41 \pm 0.08 (0.95 \pm 0.02)	7.74 \pm 0.06 (0.98 \pm 0.03)	----
p-F-HHSiD	7.76 \pm 0.08 (0.85 \pm 0.03)	6.61 \pm 0.11 (0.93 \pm 0.02)	7.90 \pm 0.01 (1.01 \pm 0.05)	7.64 \pm 0.04 (1.07 \pm 0.01)	-----
Propantheline	9.68 \pm 0.07 (1.02 \pm 0.04)	9.47 \pm 0.08 (0.95 \pm 0.01)	10.10 \pm 0.10 (1.05 \pm 0.01)	10.11 \pm 0.13 (1.07 \pm 0.02)	8.93 ^b

^a Data was adapted from Bodor et al. (1980).

^b Data was adapted from Kumar and Bodor (1996).

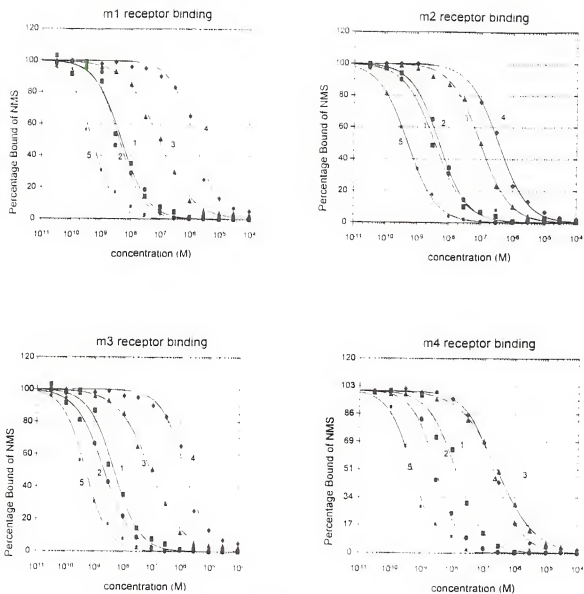


Figure 4-1. Binding isotherms of classical anticholinergics: atropine (1), scopolamine (2), F-p-HHSiD, pirenzepine (4), and propantheline (5) for the displacement of [3 H]-NMS binding to four clone muscarinic receptors.

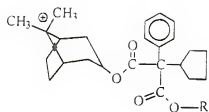
Table 4- 2. Binding parameters of soft anticholinergics at m1, m2, m3, and m4 receptors. The affinity estimates were derived from [³H]NMS displacement experiments and represented the mean (\pm S.E.M. =3-5) for the negative logarithm of K_i. The Hill coefficients are given in parentheses.

Compound	Muscarinic Subtype Receptor Binding				P _{A₂}
	m1	m2	m3	m4	
DMPC	9.25 \pm 0.04 (0.95 \pm 0.02)	8.76 \pm 0.08 (1.05 \pm 0.04)	9.14 \pm 0.06 (0.77 \pm 0.03)	9.42 \pm 0.08 (0.94 \pm 0.01)	9.3 ^a
MPC	8.48 \pm 0.08 (0.88 \pm 0.04)	8.08 \pm 0.53 (0.79 \pm 0.05)	8.58 \pm 0.07 (0.98 \pm 0.03)	8.71 \pm 0.07 (0.98 \pm 0.01)	8.4 ^a
AQC	9.07 \pm 0.02 (0.96 \pm 0.07)	9.16 \pm 0.03 (0.88 \pm 0.02)	9.20 \pm 0.05 (1.05 \pm 0.01)	8.76 \pm 0.18 (0.04 \pm 0.02)	8.55 ^a
MDP	8.60 \pm 0.03 0.98 \pm 0.05	8.71 \pm 0.01 0.91 \pm 0.07	8.57 \pm 0.08 0.87 \pm 0.05	8.25 \pm 0.05 0.97 \pm 0.02	7.95 ^a
PMTRet	7.35 \pm 0.18 (0.80 \pm 0.10)	7.39 \pm 0.06 (0.66 \pm 0.05)	8.10 \pm 0.10 (0.72 \pm 0.03)	7.78 \pm 0.20 (0.97 \pm 0.03)	7.85 ^b
PMTRCh	8.05 \pm 0.12 (0.90 \pm 0.01)	7.26 \pm 0.15 (0.78 \pm 0.05)	7.40 \pm 0.04 (1.06 \pm 0.02)	7.45 \pm 0.09 (0.95 \pm 0.02)	7.35 ^b
PMTRHx	7.39 \pm 0.03 (0.94 \pm 0.01)	6.92 \pm 0.02 (0.86 \pm 0.03)	6.82 \pm 0.02 (0.94 \pm 0.05)	6.86 \pm 0.02 (0.76 \pm 0.03)	6.40 ^b
PMSCet	7.56 \pm 0.06 (0.64 \pm 0.03)	7.13 \pm 0.04 (0.64 \pm 0.03)	7.08 \pm 0.03 (0.74 \pm 0.05)	7.28 \pm 0.03 (0.91 \pm 0.03)	7.40 ^b
PCMS-I	7.95 \pm 0.02 (0.98 \pm 0.03)	7.82 \pm 0.06 (0.98 \pm 0.03)	8.18 \pm 0.07 (0.89 \pm 0.02)	8.2 \pm 0.05 (0.88 \pm 0.02)	7.19 ^c
PCMS-II	7.24 \pm 0.05 (0.93 \pm 0.04)	7.37 \pm 0.08 (0.93 \pm 0.04)	7.34 \pm 0.03 (0.89 \pm 0.12)	7.41 \pm 0.04 (0.95 \pm 0.01)	7.02 ^c
PMMSO	7.25 \pm 0.04 (0.59 \pm 0.01)	6.90 \pm 0.10 (0.59 \pm 0.01)	7.02 \pm 0.09 (0.67 \pm 0.01)	6.91 \pm 0.18 (0.96 \pm 0.01)	7.2
PSDT	6.50 \pm 0.13 (0.56 \pm 0.21)	6.76 \pm 0.03 (0.58 \pm 0.04)	7.12 \pm 0.05 (0.50 \pm 0.03)	N/A	5.88
PMDT	7.18 \pm 0.02 (0.57 \pm 0.04)	7.79 \pm 0.02 (0.65 \pm 0.10)	7.79 \pm 0.20 (0.71 \pm 0.01)	7.72 \pm 0.10 (0.78 \pm 0.21)	7.29
S2-19	8.00 \pm 0.05 (0.96 \pm 0.01)	7.82 \pm 0.13 (0.85 \pm 0.34)	8.05 \pm 0.05 (0.68 \pm 0.04)	8.18 \pm 0.21 (0.79 \pm 0.05)	N/A
S2-21	7.57 \pm 0.08 (0.85 \pm 0.06)	7.29 \pm 0.22 (0.94 \pm 0.02)	7.73 \pm 0.08 (0.68 \pm 0.02)	7.40 \pm 0.01 (0.76 \pm 0.03)	N/A
544	6.64 \pm 0.03 (0.94 \pm 0.01)	6.45 \pm 0.10 (0.89 \pm 0.04)	6.46 \pm 0.05 (0.89 \pm 0.01)	6.90 \pm 0.09 (0.91 \pm 0.03)	---
548	7.54 \pm 0.05 (0.98 \pm 0.11)	6.94 \pm 0.04 (0.86 \pm 0.04)	7.81 \pm 0.01 (1.03 \pm 0.04)	8.02 \pm 0.04 (0.88 \pm 0.02)	----

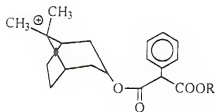
^a Data was adapted from Bodor et al., 1980

^b Data was adapted From Kumar and Bodor, 1996.

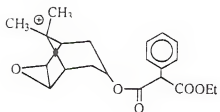
^c Data was adapted from Juhász et al., 1998.



-R	Compound
CH_2CH_3	PCMS-I
CH_3	PCMS-II

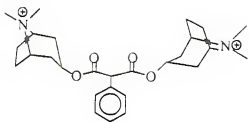


-R	Compound
ethyl	PMTRet
cyclohexyl	PMTRCh
hexyl	PMTRHx

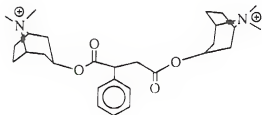


PMSCet

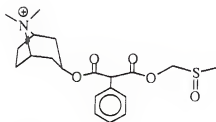
Compound



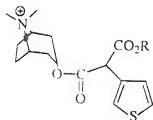
PMDT



PSDT



PMMSO



R

ethyl

52-21

isopropyl

52-19

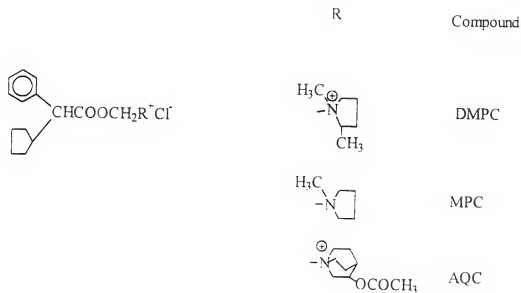
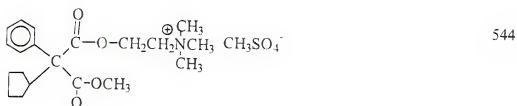
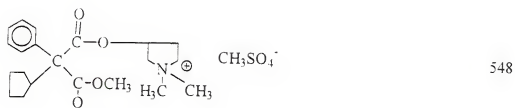


Figure 4-2. Structure of soft anticholinergics for the receptor binding studies.

Table 4-1 and Table 4-2 shows the mean $pK_i \pm S.E.M.$ values obtained for each compound in receptor binding studies. Our pK_i values of atropine, scopolamine, p-F-HHSiD (m_3 selective agent), and pirenzepine (m_1 selective agents), were in agreement with published data (Buckely et al., 1989; Dorje et al., 1991; Wess et al., 1991; Bolden et al., 1992). The Hill coefficients, n , for the above compounds were not significantly different from unity, indicating that the drug-receptor interactions obeyed the law of action and that binding was only to one site. This further validates the method we used to evaluate the binding of soft anticholinergics. However, Hill coefficients for soft anticholinergics, are significantly different from unity. Theoretically, n is an integer reflecting the number of molecules that bind to a specific drug receptor. Normally the binding of classical antagonists to muscarinic receptors is well described by the simple Langmuir isotherm, indicating a Hill coefficient close to unity (Hulme et al., 1978). It is believed that low Hill coefficients are often attributed to either recognition by the antagonist of more than one receptor site or receptor conformation, or to interaction of the antagonist with a second binding site on the receptor molecule, causing a negative cooperative effect on the first site (Nathanson, 1987; Hume et al., 1981; Barbier et al., 1995).

The cause of Hill coefficient significantly differ from unity might be related to the partial hydrolysis of the soft drugs generating structural similar inactive metabolites during incubation. The rate for the hydrolysis of soft drug in biological media is concentration dependent (Bodor et al., 1995; Yang et al., 1995). Normally, the lower the concentration, the faster the hydrolysis is. Because the hydrolysis of soft drug is mediated by esterase enzyme. Like all other enzyme involving mediating chemical and biochemical

reaction. esterase has the its capacity limitation. This is due to the fact that certain number of the active sites are existing in a fixed amount of the enzyme. When large amount of the substrates (in this case, soft drugs) are introduced into the reaction system. The active sites of the esterase enzyme are saturated. Thus, only fraction of the substrates are involving the hydrolysis reaction at one time. However, in the receptor binding studies, soft drugs were diluted to extremely low concentration (10^{-4} to 10^{-11} M). The ubiquitous esterase enzyme in receptor system might be able to reach its full capacity to hydrolyze the relative tiny amount of the soft drugs. At such condition, even fixed amount of esterase enzyme inhibitor is not able to completely prevented the hydrolysis of the soft drugs. The slightly decomposed soft drug is the reason to cause Hill coefficient smaller than 1. The metabolite resulted from hydrolysis may interfere the existing equilibrium between the receptor, the radioligand, and tested compound. Preliminary experimental data showed that the Hill coefficient (n) of AQC m_3 receptor binding was 0.4 to 0.5 when no enzyme inhibitor was added into the buffer. N increased close to unity when enzyme inhibitor (NaF) was added. The exact reason as to why n was significant different to unity is under further investigation. Searching for more effective enzyme inhibitor may improve the receptor binding methods. Our results showed that the results (pK_i) were reliable (compared with PA_2) even though the Hill coefficient did not reach unity.

It is believed that M_3 mediated smooth muscle in airway and GI tract contraction (Grimm et al.,1994). pA_2 value for guinea pig ileum contraction has been a classic functional study for the determination of anticholinergics affinity toward the M_3 receptor. The pA_2 values for soft anticholinergics generated from guinea pig ileum

contraction studies are generally comparable to pK_i value from m_3 binding studies, even though in most cases the pA_2 values are somewhat lower than the pK_i value of m_3 binding. The relative value of individual compounds tested by either method was essentially the same. In our lab, the pA_2 value of guinea pig ileum contraction was the method of choice for the screening of relative potency of soft anticholinergics (Kumar and Bodor, 1996). Because of the faster screen nature, receptor binding has an advantage over pA_2 value for providing information as to the relative potency of soft anticholinergics. The correlation of m_3 binding value and PA_2 were performed. The results were shown in Figure 4-3. Correlation coefficient was determined as $R^2=0.82$.

PMTRet, PMTRCh, and PMTRHx are soft analogs of atropine (Hammer et al., 1988; Kumar et al., 1993a). PMSCet is the soft analog of scopolamine. The design of these compounds is based on the inactive metabolite approach of soft drug design (Bodor, 1984). These compounds are expected to metabolize into hypothetical metabolites *in vivo*. Since atropine and scopolamine do not show muscarinic subtype selectivity, it is expected that these compounds will not exhibit subtype selectivity. The rank of order of potency is generally, $m_1 > m_3 > m_2$. From these three compounds, we may conclude that the substituent of the ester group has a significant impact on the binding of the soft anticholinergic to muscarinic receptors. Wess (1990) has proposed the following structural elements for cholinergic antagonists. (1) a cationic "head group" which is either a tertiary base protonated at physiological pH or quaternary ammonium moiety; (2) some "heavy blocking moieties," e.g., alicyclic or aromatic rings, for hydrophobic interaction with the receptor; (3) an interconnecting structural element (ester or amide) of definite length; (4) an "anchoring group," e.g., hydroxyl group(s) is often present at key

positions (Barlow and Ramtoola, 1980). Obviously, the differences in the structures of the above three compounds are located at the "anchor positions." Originally atropine has a hydroxyl group at the anchor position. It has been shown that replacement of the hydroxyl group with an ester group significantly decreases binding of an antagonist to muscarinic receptors. It is confirmed that the hydroxy group forms a hydrogen bond at the binding site, which is a critical element for the pharmacophore (Waelbroeck et al., 1990). It is also demonstrated that the size of the substituent has a great influence on the binding. As the size of the substituents increases, the potency decreases (Kumar et al., 1994). But there is an exception. Compared with a cyclohexyl substituent, an n-hentyl substituent has a smaller volume than cyclohexyl substituent. But n-hentyl substituent is much less potent than cyclohexyl substituent (Kumar, 1993a). It is believed that the steric hindrance of the "anchor group" (in this case, a long chain n-hentyl group) has decreased the activity. Similar results were found by Banerjee and Lien (1990), when they studied amino esters of α -substituted phenyl acetic acid, α -methyl tropic acid, and related compounds. DMPC, MPC, AQC, and MDP are soft drugs, where design is based on the soft analog approach (Bodor et al., 1980; Bodor, 1984; Brouillette et al, 1996). The soft analog approach requires a specific metabolically sensitive spot incorporated into the structure, leading to a close structural analog of known active anticholinergic drugs. The soft analogs were designed to hydrolyze *in vivo* in a predictable time-frame to an acid, an aldehyde and a tertiary amine, all inactive metabolites (Bodor, 1984). We did not find any muscarinic receptor subtype selectivity for this group of compounds.

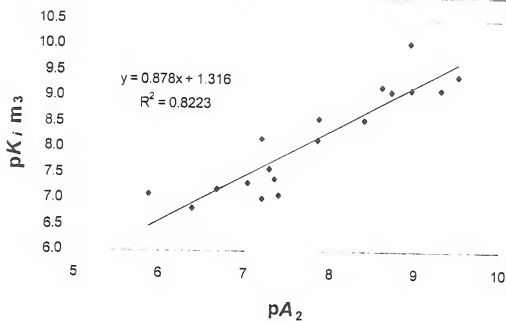


Figure 4-3. Correlation between $pK_i \text{ (m}_3)$ and pA_2

The general order of anticholinergic potency relative to the nitrogen head is, 1,2-dimethylpyrrolidine > 3-acetoxyquinuclidine > 1-methylpyrrolidine. This is in agreement with the findings of Bodor et al. (1980) and Brouillette et al. (1996).

PCMS-I and PCMS-II comprised of a group of new anticholinergics. They are quite similar to atropine analog PMTRET, except a cyclophenyl- group was incorporated into the structure. Again, this group of compounds do not exhibit subtype selectivity. Interestingly, the ethyl ester of this group is shown to be 5 times more potent than the methyl ester. Because we did not make the methyl ester soft analog of methatropine (Kumar, 1993c), we don't know this (in PCMS-II) is a unique case, where the methyl ester is less potent than the larger substituent, ethyl ester, (in PCMS-I) or it only happened when a cyclopentyl group was incorporated into the parent compound methatropine. Generally, the potency of the anticholinergics based on methatropine are inversely proportional to the molecular volume of compounds (Kumar et al., 1994).

The utilization of anticholinergics as antiperspirants has been evaluated for a long time. Scopolamine has been shown to be very effective in the inhibition of eccrine sweating (Shelley and Horvath, 1951). Other muscarinic antagonists also have been found to effectively suppress sweating (Stoughton et al., 1964; Macmillan et al., 1964; Oroshnik and Soldati, 1978). However, due to the tremendous risk of systemic toxicity when overdose, it was suggested these agents should be used under direct physician supervision and not be available for over-the-counter sale (Lasser, 1967). On the other hand, the availability of soft anticholinergics should warrant the safe use of these agents as antiperspirants, since they do not show systemic toxicity (Bodor, 1984). Obviously,

the effectiveness of topically applied antiperspirant is related to the intrinsic activity of these agents and the ability to penetrate the skin. Binding and functional studies on muscarinic receptors of secretory cells have demonstrated that all muscarinic receptors in granular cells appear to be of the M_3 subtype (Goyal, 1989). The results of our data have shown that PMTRET, AQC, and DMPC have very high affinity toward m_3 receptor. Kumar (1993c) has found a linear correlation between the log partition coefficients ($\log K_p$) and log permeability coefficients ($\log p$) of the soft anticholinergics tested. The $\log K_p$ can be readily estimated by the a semiempirical quantum chemical method (AM1) (Bodor et al., 1989; Bodor et al., 1992). It should be easy to screen the most promising candidates for a safe antiperspirant by combining the m_3 binding data and $\log K_p$ values obtained from computer estimations. Our data showed the m_3 binding values are comparable to functional studies on guinea pig ileum. It has long been regarded muscarinic receptor on smooth muscles are of M_3 subtype. The latest research have demonstrated that the muscarinic receptors in guinea-pig ileum are heterogeneous, with a major M_2 receptor population (~80%) and a minor M_3 population (~20%). The function of the minor M_3 population is clearly related to contraction, but the function of the predominate M_2 population is unclear. It may be related to the inhibition of relaxation of the muscle (Eglen et al., 1994). Therefore, utilization of receptor binding data of m_3 for the *in vitro* estimate of inhibition of eccrine sweating should be more accurate than that of functional studies based on smooth muscles contraction.

Quantitative structure activity relationship (QSAR) studies were carried out to investigate the relationship of physicochemical parameters with receptor binding values. It was found the following formula to describe the QSAR:

$$pK_i (m_3) = 22.747(\pm 2.700) - 6.728(\pm 1.357)O_e - 0.104(\pm 0.025)D$$

$$n = 20, r = 0.843, \sigma = 0.426, F = 20.90$$

It showed that for soft anticholinergic agents containing a tropine moiety, pK_i (m_3) values correlated well with molecular ovality (O_e) and dipole moment (D). Consequently, receptor binding for this group of compounds in addition to overall shape and size (O_e) is determined by electronic properties (D) as well. The correlation between predicted and experimental values is shown in Figure 4-4.

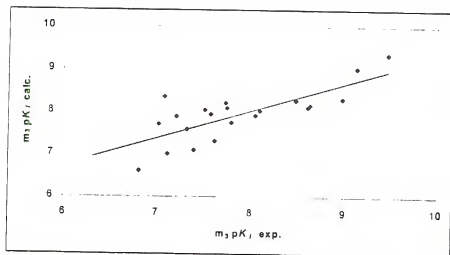


Figure 4-4. Calculated versus experimentally measured $m_3 pK_i$ data for inactive metabolite-type soft compounds containing a tropine moiety.

In conclusion, the binding experiment performed on cloned muscarinic receptors provided valuable affinity information about soft anticholinergics toward individual muscarinic subtype. The information will assist in the study of the structure activity relationship and screening the ideal candidates for the development of soft antiperspirants and soft mydriatics. This information will also assist us in the development of soft anticholinergics with muscarinic subtype selectivity. This should further increase the therapeutic index of soft anticholinergics.

Receptor Binding Data for the New Class of Soft Anticholinergics

Receptor binding studies were performed on the newly synthesized soft anticholinergics 9(a-b) and 13 (a-b). The results were listed in Table 4-3. The newly synthesized soft anticholinergics are able to attain the potency of the lead compound. It is also demonstrated that the newly synthesized compound 9a and 13a showing muscarinic subtype selectivity (m_3/m_2). In addition, compound 9a also had m_3/m_1 selectivity.

From the QSAR studies, Turbanti et al. (1992) proposed that the smaller the size at equatorial N-substituent of N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acids, the more potent the compound. According to their proposal, 13a of our series should be much more potent than other soft anticholinergics. Actually, this was not case. There is not a significant difference in the potency

Table 4-3. Receptor binding values for 9(a-b) and 13(a-b). The affinity estimates were derived from [^3H]NMS displacement experiments and represented the mean (\pm S.E.M, $n=3-5$) for the negative logarithm of K_i . The Hill coefficients are given in parentheses. To ensure the experimental conditions are consistent, the receptor binding values of atropine were determined simultaneously with soft anticholinergics at each experiment.

Compound	m1	m2	m3	m4
atropine	9.08 \pm 0.12 (0.98 \pm 0.03)	9.04 \pm 0.20 (1.01 \pm 0.02)	9.28 \pm 0.07 (0.96 \pm 0.02)	9.50 \pm 0.04 (1.01 \pm 0.04)
9a	7.86 \pm 0.03 (0.78 \pm 0.05)	7.73 \pm 0.10 (0.91 \pm 0.10)	8.99 \pm 0.01 (0.81 \pm 0.02)	8.43 \pm 0.07 (0.90 \pm 0.02)
9b	7.93 \pm 0.04 (0.86 \pm 0.01)	7.97 \pm 0.03 (0.88 \pm 0.03)	8.64 \pm 0.05 (0.87 \pm 0.06)	8.20 \pm 0.06 (0.91 \pm 0.07)
13a	7.89 \pm 0.07 (0.83 \pm 0.05)	7.38 \pm 0.07 (1.07 \pm 0.07)	8.49 \pm 0.02 (0.81 \pm 0.04)	8.11 \pm 0.06 (1.07 \pm 0.02)
13b	7.98 \pm 0.04 (0.91 \pm 0.05)	7.70 \pm 0.06 (0.80 \pm 0.06)	8.62 \pm 0.05 (0.87 \pm 0.05)	8.17 \pm 0.03 (0.82 \pm 0.05)
^a lead	8.20	7.47	8.64	N/A

^a: data were adapted from D'Agosting et al., 1994.

between methyl- or ethyl- ester soft anticholinergics. There is not a significant difference in the potency between α and β isomers.

Muscarinic receptors are involved in the control of functions of many organs in the body. Three major muscarinic receptor subtypes: M_1 , M_2 , and M_3 mediate a variety of basic function of the body (for review, see Introduction). These receptors have a specific location and control a particular physiological activity. However, most of the currently available anticholinergic agents are not subtype selective agents. Such anticholinergics should equally stimulate all muscarinic subtype receptors in the body once they are brought into systemic circulation. Thus, a therapeutics for an intended symptom usually result in many undesired effect. The usefulness of the anticholinergic agents are limited due to their relatively low selectivity. It is not difficult to understand why the available anticholinergic agents have very low subtype selectivity once we examine the genetic structure the muscarinic receptors. The human genes which encode five muscarinic subtype receptor have 70% similarity in their sequence (Bonner et al., 1987). The three dimensional structures of the receptor, critical factor determining the selectivity of the receptor, are based on the sequence of the genes. In developing a receptor to perfectly fit into a subtype receptor, there is a very good chance it will fit into other subtype receptors. However, the need to develop muscarinic subtype selective agents is great. The M_1 -selective antimuscarinic agents can be used to inhibit gastric secretion and is applied in the treatment of peptic ulcer disease (Goyal, 1989). Cardiac M_2 receptor mediate bradycardia. M_2 -selective antagonist could be used to the therapy of bradyarrhythmias. The M_3 receptor selective anticholinergic agents could be used as bronchodilators in the treatment of chronic obstructive pulmonary diseases. They can also be used as safer

premeditative agents for the reduction of the secretory effects of anesthesia, at the same time minimizing the cardiac effects of the traditional premeditative agents, such as atropine and glycopyrrolate (Brown and Taylor, 1996). For soft anticholinergics with subtype selectivity (M_3/M_2), they possess the safer feature of the soft drugs, that is, locally active but systemically inactive. This is the pharmacokinetic and metabolism approach to increase the therapeutic index. Besides the safer feature from pharmacokinetic consideration, this new class of soft anticholinergics holds unique properties: subtype selectivity. It is certainly a pharmacodynamic approach to enhance the therapeutic index. As our earlier discussion, such an approach would not be able to completely ensure the safety of the agents because of genetic similarity of the subtype muscarinic receptors. It is obvious, if the goal of the design of new therapeutics is to maximize the therapeutic index, the inclusion of consideration of both pharmacodynamic and pharmacokinetic properties in the drug design would greatly speed up the drug discovery process and produce much safer and effective agents. The possible clinical use of the soft anticholinergics with subtype selectivity (M_3/M_2) are as follows. (1) Mydriatic agents: we have mydriatic studies to demonstrate such usefulness. (2) Antiperspirants. Anticholinergics have been explored as antiperspirants for a long time (MacMillan et al., 1964; Stoughton et al., 1964). However, the potential side effects prevented their use for this purpose. The development of the subtype selective quaternary soft anticholinergics would certainly reduce the risk of the cardiac effects and CNS toxicity. (3) Anticholinergics, such as atropine and glycopyrrolate, are frequently used for premedication to reduce oral and respiratory secretions and prevent bradycardia (Brown and Taylor, 1996).

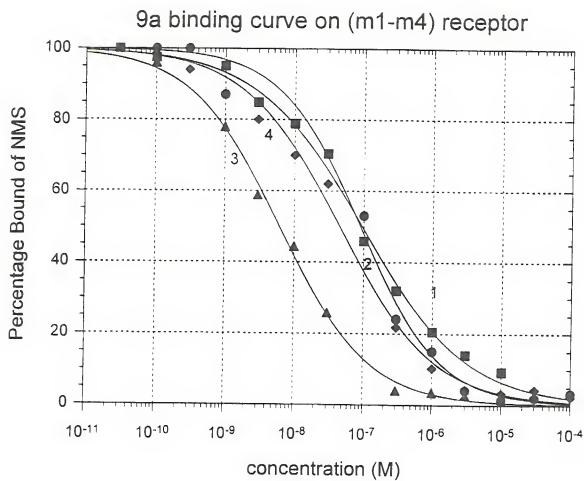


Figure 4-5. Binding isotherms of 9a for the displacement of specific [³H] NMS binding to m1 (1), m2 (2), m3 (3), and m4 (4) muscarinic receptors.

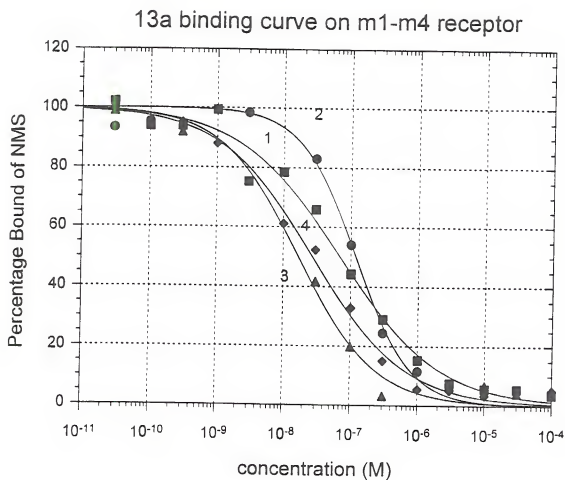


Figure 4-6. Binding isotherms of 13a for the displacement of specific [^3H] NMS binding to m1 (1), m2 (2), m3 (3), and m4 (4) muscarinic receptors.

They are also administered with neostigmine for the reversal of non-depolarizing neuromuscular blockage (Wetterslew et al., 1991; Mirakhur et al., 1977; Takkunen et al., 1984). However, such application has been complicated by the cardiac side effects of the anticholinergics, even though glycopyrrolate causes less cardiac side effects (Gomez et al., 1995; Wetterslew et al., 1991; Mirakhur et al., 1977; Takkunen et al., 1984). This is particularly dangerous to the patients with pre-existing cardiac disease (Mostafa and Vucevic, 1984). The development of soft anticholinergics with subtype selectivity (M_3/M_2) will assist the safer administration in anesthesia; either as premeditation for reducing excessive salivation and secretions of the respiratory tract induced by administration of general anesthetic agents or as an agent to reverse the non-depolarizing neuromuscular blockage. The occasional serious arrhythmias effects associated with agents (Brown and Taylor, 1996) should be greatly reduced due to subtype selectivity (M_3/M_2). The soft nature of the compounds will allow us to add the exact amounts of anticholinergics needed during the anesthesia practice by titration.

Receptor binding studies of soft anticholinergics based on tropyl α -phenyl cyclopeneacetate

Receptor binding studies were performed on the newly synthesized soft anticholinergics based on tropyl- α -phenylcyclopeneacetate. The results were listed in Table 4-4. The results showed that the P_{ki} 's of methyl soft drugs (15a and 18a) are higher than that of ethyl soft drugs, indicating the methyl soft drug were relatively potent than ethyl counterpart. It is in agreement with the previous finding from our laboratory (Kumar et al., 1994; Juhasz et al., 1998) that the smaller the molecule size, the more

Table 4-4. Receptor binding studies: The numbers of the table stand for pKi (Ki: dissociation constant, data were the mean of three determinations). The higher the pKi, the more potent the compounds.

Compounds	m1	m2	m3
15a	7.65 ±0.01 (0.83 ±0.04)	7.54±0.18 (0.78±0.02)	7.75±0.10 (0.73±0.01)
15b	7.42 ±0.04 (0.87 ±0.02)	7.20±0.03 (0.83 ±0.04)	7.57±0.08 (0.75±0.01)
18a	7.33±0.10 (0.76±0.03)	7.14±0.06 (0.85±0.07)	7.51±0.15 (0.80±0.06)
18b	7.00±0.08 (0.88±0.05)	6.94±0.09 (0.85±0.07)	7.21±0.2 (1.01±0.03)

potent the compound. The β isomer has somewhat higher affinity toward muscarinic receptors.

In Vivo Activity- Mydriatic Studies

Firstly, the dose and mydriatic response relationship was established by administering increasing concentrations of the compounds until the maximum dilation was achieved. Due to mechanical restriction, the pupil will not dilate infinitely. The lowest dose that produces the maximum achievable dilation was used as the dose for comparison. Atropine (0.3% w/v), tropicamide (0.33% w/v), 13a (0.3% w/v), 13b (0.5% w/v), 9a (0.5% w/v), 15a (1% w/v), 18a (1% w/v), and 18b (2% w/v) produced equieffectiveness. 9b and 15b had very low solubility, they were not studied. Most of these compounds show non-irritation. Only compound 9a showed mild to moderate irritation. The maximal dilation was observed in one hour after administration without significant difference among soft drugs, atropine, and tropicamide. In order to adequately compare the duration of the mydriatic action of soft anticholinergics with atropine and tropicamide, the area under response curve of 24 hours (AUC_{24hr}) was calculated with trapezoidal rules for each compound of each trial. The results were shown in Figure 10. The AUC_{24hr} of 13a, 15a, and 18a are significantly smaller than that of tropicamide ($P < 0.05$). The AUC_{24hr} of 13a, 13b, 15a, 18a, and 18b are significantly smaller than that of atropine sulfate ($p < 0.05$). The time course of mydriatic activity of the treated eye were depicted in Figure 4-7 and Figure 4-8. The recovery time for atropine, tropicamide, 9a, 13a, 13b, 15a, 18a, and 18b are 24hr, 10hr, 20hr, 7.5hr, 8.5hr, 8.5hr, 11hr, and 11hr,

respectively. From the AUC_{24hr} and recovery time of soft anticholinergics, atropine, and tropicamide, we can conclude that the duration of mydriatic action of 13a, 13b and 18a are shorter than that of tropicamide, the most frequently used mydriatic agents currently in the market. In our studies, the recovery time of tropicamide was 10 hours, which is different from the published data: 6 hours (Hammer et al., 1991; Kumer et al., 1993b). It might be due to the physiological difference of the rabbits. At the equieffective dose, all soft anticholinergics tested, except 9a, showed significantly shorter duration of mydriatic action in the treated eye than atropine sulfate. Generally, the methyl esters are shorter acting than that of ethyl esters, the α isomers are shorter acting than that of β -isomers. Deshpande and Schoenwal (1998) studied the methyl, ethyl, and propyl esters of ethacrynic hydrolyzed in the rabbit ocular tissue (cornea and iris-ciliary body homogenates). They found that methyl esters were hydrolyzed fastest, followed by ethyl esters and then propyl esters. The lowest concentration needed to achieve the maximum pupil dilation was generally in agreement with the receptor binding data. For 9a, 13a, and 13b, the m_3 receptor binding value is virtually the same ($9a=8.97$, $13a=8.53$, $13b=8.71$), the lowest concentration required to reach maximum pupil dilation is virtually the same ($9a=0.5\%$, $13a=0.3\%$, $13b=0.3\%$).

For 9a, even though its binding value (m_3 , $P_{ki}=8.97$) is little higher than that of 13a and 13b, it needed a slightly higher concentration to achieve the maximum pupil dilation. It could be owing to its β - isomer structure which may be unfavorable to bind to the receptor in pigment or because of its counter ion, Br^- , which makes the solubility of the compound lower, compared with methyl sulfate. It may reduce its ability to penetrate the cornea membrane.

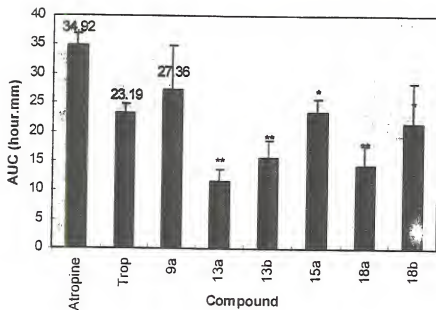


Figure 4-7. Comparison of AUC_{24hr} of soft anticholinergics with AUC_{24hr} of atropine and tropicamide. * indicating AUC_{24hr} is smaller than that of atropine only ($p < 0.05$); ** indicating AUC_{24hr} is smaller than that of atropine sulfate and tropicamide ($p < 0.05$).

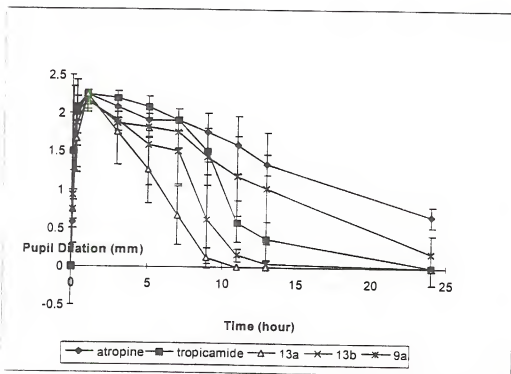


Figure 4-8. Time course of mydriatic response (treated eye) for atropine sulfate, tropicamide, 13a, 13b, and 9a.

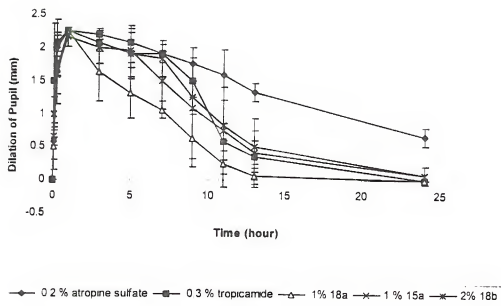


Figure 4-9. Time course of mydriatic response (treated Eye) for atropine sulfate, tropicamide, 18a, 15a, and 18b.

For 15a, 18a, and 18b, the pK_i (m_3) is 7.86, 7.23, and 6.86, respectively, and the lowest concentration needed to achieve the maximum is 1%, 1% and 2%. The *in vitro* and *in vivo* activities correlated very well.

The soft anticholinergics, except 9a, are found to be none to mild irritation on the topical administration into the eye. There are reports in the literature which suggested the relationship between hydrophobic side chains and irritation potential of pharmaceutical compounds (Fraunfelder, 1989). Because the soft anticholinergics tested are generally shorter side chains (methyl and ethyl group), it may cause less irritation. The time course of mydriatic activity in the untreated eyes (at the equieffective dose) are depicted in Figure 4-9 and Figure 4-10. Significant dilation of the untreated eyes was observed with atropine sulfate and tropicamide, but not with soft drugs. It means that the "hard drug" atropine and tropicamide are able to dilate the untreated eye after systemic absorption. For soft drugs, the systemic absorbed compounds would undergo a one step deactivation to inactive metabolite, so the systemic toxicity was minimized. More than 90% of the topically administered drugs have been reported to be drained into systemic circulation through nasolacrimal duct without entering the interior of the eye. This results in a high incidence of systemic side effects after ocular administration of drugs. Any potential toxic systemic effects that would be a result of the intact soft drugs' absorption into the systemic circulation should be less than those for other currently available mydriatic drugs because the quaternary nature of the soft drug prevents its passage across the blood-brain barrier into central nervous system, and the subtype selectivity (m_3/m_2)

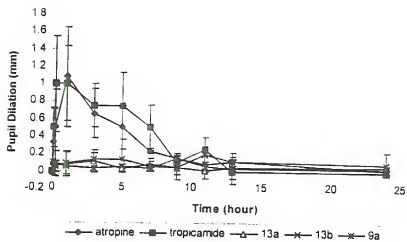


Figure 4-10. Time course of mydriatic response (control Eye) for atropine sulfate, tropicamide, 13a, 13b, 9a after unilateral administration.

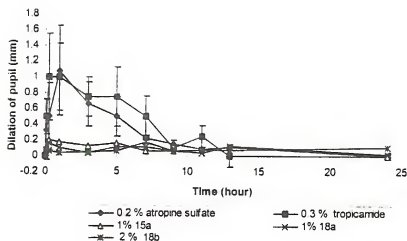


Figure 4-11. Time course of mydriatic response (control eye) for atropine sulfate, tropicamide, 15a, 18a, and 18b after unilateral administration.

would reduce its cardiac toxic effect. And its one-step deactivation would lead to a less active, more polar quaternary metabolite that would be rapidly excreted.

In vivo Pharmacodynamic Evaluation—Cardiac Studies.

We evaluated cardiac effect of soft anticholinergics by measuring the extent and duration of action of the bradycardia protective effect of soft anticholinergics. With the i.v. administration of carbachol at a dose of 5-8 μM (27 - 44 $\mu\text{mol/kg}$) to male Sprague-Dawley rats, the temporary development of sinus bradycardia and Mobitz II A-V block can be evoked safely and repeatedly. This effect can be antagonized by the previous administration of an anticholinergic agent, e.g., atropine, scopolamine, glycopyrrolate, and propantheline. The full protection against carbachol induced bradycardia by the anticholinergics was regarded as their ability to protect against both the lengthening of the PP cycle (sinus bradycardia) and the development of the Mobitz II type A-V block. The bradycardia protective effects of the different anticholinergics differ greatly in respect of their potency and duration of action. In preliminary experiments, a duration of action longer than two hours was found for atropine methyl bromide (atropineMeBr). In order to compare the duration of the soft anticholinergics and atropine, the approximate pharmacodynamic equivalent dose was administered into Sprague-Dawley rats. Three compounds, 9a (2 μM), 13a (2 μM), and 15a (0.2 μM and 2 μM) were tested. appearance of Mobitz II A-V block only in 10 minutes. 15a has such effect up to 45 minutes. 9a showed longer protective effect than that of 13a, which is similar to

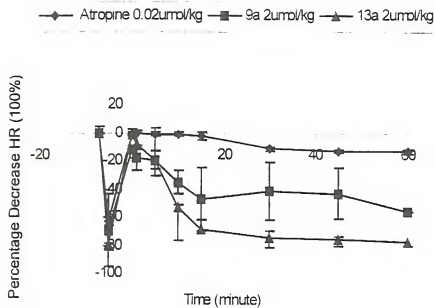


Figure 4-12. Bradycardia protective effects of 9a, 13a, and atropine MeBr as illustrated by the percentage change in the heart rate.

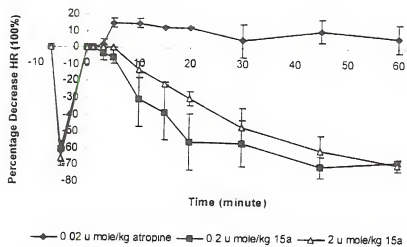


Figure 4-13. Bradycardia protective effect of 15a and atropineMeBr as illustrated by percentage heart rate change.

mydriatic studies. This means that the α isomers is hydrolyzed faster than the β isomers. In mydriatic studies, the recovery time for 9a ($AUC_{24hr}=27.36$) is much longer than 13a ($AUC_{24hr}=11.52$). Juhasz et al. (1998) has reported studies of duration of antagonism of carbachol-induced bradycardia of several soft anticholinergics based on tropane analogs. They found that PCMS-II, a soft drug with introduction of bulky cyclopentyl group has a duration time of 15-30 minutes. 9a, 13a, and 15a have the similar structures of PCMS-II, and they have the similar duration. On the other hand, the compound without such bulky group, tematropium sulfate has a duration of 3 minutes (Bodor et al., 1990). It is suggested that the introduction of bulky group into the moieties would hinder the hydrolysis of the ester soft drug.

Pharmacokinetic Studies--- *In Vitro* Biotransformation Studies

Analytical procedure

An accurate and reproducible qualitative and quantitative method of analysis is needed for the estimation of compounds and their degradation and metabolic products. The high performance liquid chromatography (HPLC) has been the most widely used method for such purposes. The main advantages of HPLC are its reproducibility, accuracy of quantitation, and economy of operation. In our research, we used reversed-phase chromatography (RP-HPLC). RP-HPLC is the most widely used chromatographic mode used to separate neutral molecules in solution on the basis of their hydrophobicity. The analysis of strong acids or strong bases using reversed-phase columns is typical accomplished by the technique known as ion-pair chromatography. In this technique, the

pH of the eluent is adjusted in order to encourage ionization of the samples: for acids pH 7.5 is used, and for bases pH 3.5 is common. Retention is then altered by including in the mobile phase a bulky organic molecule having a charge opposite from that of the ion to be analyzed. The counterion is the ion-pairing reagent. The samples we analyzed were quaternary ammonium salts, acetic acid was added to encourage ionization of the samples. The ion-pairing agent we used was 1-octanesulfonic acid. The retention time for the compounds was 3 to 8 minutes, which was variable as the changing of the contents of acetonitrile and water. The metabolites are more polar than the corresponding compounds, they are eluted faster than the parent compounds. The recovery rate for the compounds in the biological media (rat blood, rat plasma, rat liver homogenate) are > 97%. In the HPLC system, the concentration vs the area under the peak plot showed linearity ($r=0.99$) for the range of 0.5 μg to 10 μg of the injected sample with a detection limit of 0.5 $\mu\text{g/ml}$ at the sample injection volume of 40 μl . The absorbance for the compounds 9a, 9b, 13a, and 13b is higher than compounds 15a, 15b, 18a, and 18b, because one more double bond was in the acid part of the moiety of the first series of the compounds. The U.V. absorbance was set at 254nm.

In vitro stability studies.

The enzymatic target of the soft drugs is the ester functionality which is expected to undergo rapid, one step hydrolyses by the reaction with blood and tissue esterase. To prove that the soft drugs possess appropriate properties for the clinical application, the stability and metabolic pathways in blood and liver homogenate obtained from rats was investigated. The rates of disappearance of the drugs were used to calculate their $t_{1/2}$

values. *In vitro* stability studies in biological matrices may not result in absolute values but will give a relative picture of the stability profile of the compounds. They also give clues to the possible metabolic profile in the *in vivo* conditions. In the biological media, the metabolism of the soft anticholinergics was a pseudo first-order kinetics, and all soft drugs were converted to the acid metabolites. The metabolites were not synthesized. However, in all soft drugs investigated, there is only one more peak with higher polarity in the HPLC chromatogram after *in vitro* biotransformation studies for all compounds compared with the original soft drug in the chromatogram. Presumably, they are the metabolites. As shown from Table 4-5 and Table 4-6, the methyl ester is more rapidly hydrolyzed than the ethyl ester of soft drugs. The rate of hydrolysis of α -isomers are similar to that of β isomers. This once again showed the general trend that the longer and the more steric hindered the side chain, the longer the hydrolytic half life (Hammer et al., 1988; Kumar 1992; Deshpande and Schoenwald, 1998). The hydrolytic rates were higher in rat plasma than in rat whole blood, probably indicating that erythrocyte binding of the compounds is significant. The overall results of the *in vitro* biotransformation studies of the compounds demonstrated that the "soft" acyloxyalkyl linkage provides a metabolically sensitive spot which allowed the facile decomposition of the soft analogs to the inactive moieties.

Table 4-5. *In vitro* stability of compounds 9a, 9b, 13a, and 13b. Data are half life of the compounds in biological media (min). They are the mean of three determination.

compound	rat plasma	rat blood	rat liver homogenate
9a	15.10	136.33	101.05
9b	20.90	185.92	108.01
13a	28.80	83	89.54
13b	30.59	192.00	107.30

Table 4-6. *In vitro* stability of compounds 15a, 15b, 18a, and 18b. The data are half life of the compounds in biological media (min). They are the mean of three determination.

compounds	rat plasma	rat blood	rat liver homogenate
15a	23.64	171.35	186.60
15b	38.27	285.92	274.89
18a	17.48	185.85	179.24
18b	29.04	201.05	275.98

In Vivo Pharmacokinetic Studies- II' Bolus Studies of 13a.

In vivo pharmacokinetic studies were performed on one of these compounds, 13a, in rats. The concentrations of the compound were determined by HPLC. Due to the low detection limit of the HPLC system, high doses were applied for the pharmacokinetic studies. After intravenous administration of the various doses of 13a in rats, blood concentration-time curves were developed for the pharmacokinetic evaluations. As shown in Figure 4-10, 13a was eliminated from the blood in a bi-phasic manner. The data shown in Figure 4-10 were analyzed by noncompartmental and compartmental methods. The resulting pharmacokinetic parameters are listed in Table 4-7. The concentration-time curves were very well described by an i.v. bolus two-compartmental model according to a bi-exponential equation, $C = Ae^{-\alpha t} + Be^{-\beta t}$. The statistics on the correlation coefficient of variation, >0.995 , and the model selection criterion, ranged 3.8-5.7, indicate the goodness of fit. As Table 4-7 displayed, the half life for 13a, ranged from 15 minutes to 59 minutes, showing a dose dependent manner. The elimination constants for dosage of 5 (mg/kg), 10 (mg/kg), and 15 (mg/kg), are 0.23, 0.12, and 0.11, respectively. The total clearance for a dose of 5 (mg/kg), 10(mg/kg) and 15 (mg/kg) are 16.31 (ml/min/kg), 10.21 (ml/min/kg), and 12.22 (ml/min/kg), respectively. All these data demonstrated that the elimination of 13a shows some form of dose-dependent manner. For the dose 10 mg/kg and 15 mg/kg, the elimination constant and clearance are not significantly different from each other. But, in the case of 5mg/kg dose, all these parameters are significantly different from that of 10 mg/kg and 15 mg/kg.

Table 4-7. Pharmacokinetics of 13a after intravenous bolus administration in rats.

Dose, mg/kg	5 (n=3)	10 (n=4)	15 (n=4)
AUC, $\mu\text{g}\cdot\text{min}/\text{ml}$	306.95 ± 11.89	1001.39 ± 82.35	1230.19 ± 63.08
Cl_{tot} , $\text{ml}/\text{min}/\text{kg}$	16.31 ± 0.63	10.21 ± 0.96	12.22 ± 0.65
MRT, min	16.86 ± 0.93	56.88 ± 6.55	72.66 ± 12.49
Vd_{ss} , ml/kg	274.60 ± 4.59	577.48 ± 54.65	896.53 ± 205.15
A, $\mu\text{g}/\text{ml}$	60.27 ± 15.79	115.61 ± 30.56	117.33 ± 12.38
B, $\mu\text{g}/\text{ml}$	11.46 ± 0.43	12.05 ± 1.33	12.89 ± 2.28
α , $1/\text{min}$	0.95 ± 0.175	0.73 ± 0.24	0.68 ± 0.09
β , $1/\text{min}$	0.045 ± 0.05	0.014 ± 0.001	0.012 ± 0.001
$t_{1/2}(\beta)$	14.64 ± 0.99	47.50 ± 4.23	59.52 ± 9.0
Vd_c , ml/kg	73.05 ± 15.64	90.16 ± 21.64	116.64 ± 13.10
Vd_{area} , ml/kg	365.48 ± 26.57	698.26 ± 69.00	1045.16 ± 167.72
k_{el} , $1/\text{min}$	0.23 ± 0.04	0.12 ± 0.025	0.11 ± 0.001

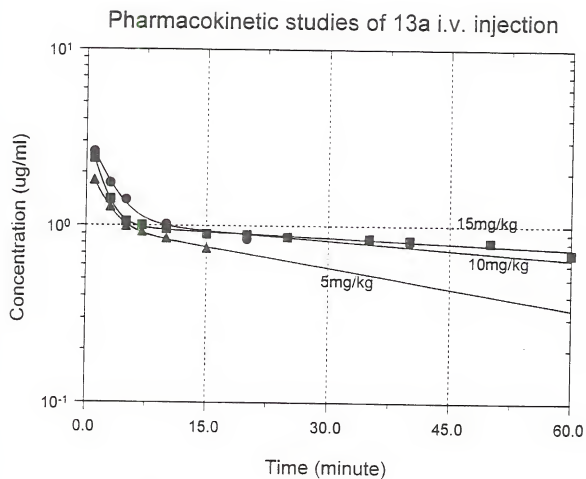


Figure 4-14. Pharmacokinetic studies of 13a by intravenous injection at the 5mg/kg, 10mg/kg, and 15mg/kg.

It means at the higher dose, the elimination of 13a was somewhat saturated. Bodor et al. (1995) found similar results when they studied the pharmacokinetics of Loteprednol Etabonate, a soft corticosteroid, in rats. Anticholinergics, especially, atropine analog, are eliminated predominately through renal excretion. The soft drug, by design, would be hydrolyzed by ubiquitous esterase in plasma. It is well known that any enzyme-mediated reaction has the capacity limitation due to the saturable nature of the active site of enzymes. As the highly concentrated soft anticholinergic agents were introduced into the systemic circulation by i.v. bolus, the hydrolyzing capacity of esterase was saturated, leading to the elimination of higher concentration of the original compound is slower than the lower concentration of the original compounds, thus, the nonlinear pharmacokinetics occurs. However, in the clinical setting, either as mydriatics or as antiperspirants, the anticholinergic agents are administered at a lower dose, e.g. 0.1 mg/kg (Brown and Taylor, 1996). Such a lower concentration would not be possible to saturate the esterase active site in systemic circulation, the soft anticholinergics are expected to fast hydrolyze into inactive metabolites so that the "local active and systemic inactive" safer agents are realized.

The clearance of 13a was 10 to 16 ml/min/kg, which is much smaller than 58 ml/min/kg reported by Urso et al. (1991), when they studied the pharmacokinetics of atropine after i.v. bolus injection in rats with RRA. The difference of the pharmacokinetics may be due to the different method for the determination of the concentration of atropine or alkaloid tropane. Our method of determination of the alkaloid tropane was HPLC, which belongs to chromatographic methods. It detects both 1-

and d- isomer of the compounds. However, the radioreceptor assay (RRA) can only detect the pharmacological active agents, l-hyocyanine. This resulted in a lower plasma concentration, a lower AUC, and higher clearance (Aaltonen et al., 1984; Kentala et al 1990; Thiemann et al., 1996) and apparent volume of distribution.

CHAPTER 5 CONCLUSIONS

Anticholinergic agents have been used in clinical practice for many years. However, their usefulness is limited due to the broad side effects caused by indiscriminate activation of all subtype muscarinic receptors in the body. To overcome such a problem, two approaches were applied to design safer anticholinergics. (1) The pharmacodynamic approach: through quantitative structure activity relationship studies of the existing compound, in conjunction with molecular biological studies of the conformation of receptors, one might be able to develop subtype selectivity anticholinergic agents, which is able to preferentially activate desired subtype muscarinic receptors. (2) The pharmacokinetic approach, through the soft drug idea, to design a local active but systemic inactive anticholinergic agent, which should be a safer local agent.

The present study involves the development of a new class of anticholinergics which are soft and subtype selective agents. In order to achieve our goals, a reliable receptor binding methods was developed for the determination of the soft anticholinergics in cloned human muscarinic receptor subtypes. The validation of the binding method was carried out by testing some of the most frequently used anticholinergics, e.g. atropine, scopolamine, pirenzepine, p-F-HHSiD, and propantheline. The results showed the methods were reliable and reproducible. The newly developed receptor binding

methods were applied to determine the potency of the soft anticholinergics made in the Center for Drug Discovery at the University of Florida. Binding data showed strong correlation with the PA_2 , a classical method for the determination of the anticholinergic potency. The Hill slope generated from the receptor binding studies of soft anticholinergics are somewhat less than 1. The addition of esterase enzyme inhibitor has improved the value close to unity. Quantitative structure activity relationship (QSAR) studies were carried out to investigate the relationships of physicochemical parameters and the receptor binding values. It was found that receptor binding value (pKi) correlated well with molecular ovality (O) and dipole moment (D) for the compounds contain a tropine moiety. To include all soft anticholinergics, such observation was not valid.

To design a new class of soft anticholinergics with subtype selectivity, N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acid were chosen as the lead compounds. Inactive metabolite soft drug design approach was adopted to the design of this new class of soft anticholinergics. A hypothetical metabolite was used as the starting compound. Two isomers (α , β) were considered for the designing due to the suggestion from literature that one of the isomers (α) showed much higher potency than another one. Another series of soft anticholinergics were also designed based on tropyl α -phenylcyclopenteneacetate.

The synthesis of these two series of soft anticholinergics involved multiple steps. Eight compounds were synthesized. The structure of the compounds were identified by NMR, mass spectroscopy, and elemental analysis.

Receptor binding studies were carried out on these two series of soft anticholinergics. Compound 9a and 13a showed subtype selectivity (M_3/M_2). All these

compounds are able to attain the potency of the lead compounds. The potency of the soft drugs based on trotyl- α -phenylcyclopeneacetate is related to the molecular volume of the compounds. The potency decreased with increased volumes.

The mydriatic activity of this new class of soft anticholinergics was evaluated in rabbit eyes. At the equieffective dose, all soft drugs showed shorter duration than atropine. Soft drugs, 13a, 13b, and 18a exhibited shorter duration than tropicamide, the shortest acting mydriatic agent available on the market. A significant dilation of untreated eye after unilateral topical administration of atropine and tropicamide were observed. In the soft drug treated animal no dilation of the untreated eye was observed. The absence of dilation in the untreated eye of soft drug treated animals indicated the facile metabolism of the soft drug in systemic circulation compared to the persistence of atropine and tropicamide.

Cardiac activity of the soft drugs were evaluated by the ability to antagonize the carbachol-induced bradycardia. 13a, 9a, and 15a were chosen to study in rats. It was shown that the atropine was able to antagonize such activity for at least two hours; however, the newly synthesized soft anticholinergics displayed such ability for a much shorter time. Compounds 13a, 9a, and 15a displayed analogizing ability in 15, 30 and 45 minutes, respectively. This further demonstrated that the soft anticholinergics were rapidly degraded *in vivo* to an inactive metabolite. Thus, the systemic activity of the soft drug should be much shorter than that of the traditional soft anticholinergics.

The *in vitro* stability of the soft anticholinergics were carried out in rat plasma, rat whole blood, and rat liver homogenate. All the soft anticholinergics showed shorter half life in the biological media than conventional anticholinergics. This showed that the less

steric hindrance of the compound, the faster the hydrolysis rate. The *in vivo* pharmacokinetic studies were carried out in one of the soft anticholinergics, 13a. The intravenous bolus injection of different doses of 13a into rats. The concentration and time curve was displayed and related pharmacokinetic parameters were calculated. It was found that the 13a displayed some form of nonlinear pharmacokinetic character. At the low dose, 13 had a very short half life of 15 minutes. It was again shown that the soft anticholinergics would be rapidly eliminated from the body if they were systemically absorbed from the local administration.

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